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# Specific Lytic Reaction of Bovine Erythrocytes Sensitized with Tuberculin, and Its Diagnostic Value upon Tuberculosis

By

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Serological methods for the detection of antibody in the sera of tuberculous patients have been often tested, but the result has only ascertained that the complement fixation test has a supplementary diagnostic value. In 1948, Middlebrook and Dubos[1] proved a hemagglutination in tuberculous sera, using sheep erythrocytes sensitized with polysaccharide fraction of human tubercle bacilli ($H_{37}Rv$). Subsequently, Smith and Scott,[2] who pursued the work of Middlebrook and Dubos, found that the sheep erythrocytes sensitized with old tuberculin by the method of Middlebrook also showed a specific hemagglutination reaction with the sera of tuberculous patients. Later, this fact was further tested repeatedly by many authors, such as Fisher and Keogh,[3] Rothbard *et al*,[4] Gernes-Rieak and Tacouet,[5] Sohier,[6] Thalheimer and Roive,[7] Boyden,[8] Kirby *et al.*,[9] Scott and Smith,[10] and also in Japan, by Yaoi *et al*[11][12][13][14] Haneda *et al.*,[15] Yoshida and Takayama,[16] and Koji.[17] Some of them further discussed the correlation of this reaction between several clinical symptoms.

Nezu and Akiba,[18] on the other hand, suggested a satisfactory result of the specific hemolytic reaction, which is introduced by adding of the complement to the mixture of immune serum which was elicited by injection of pertussis bacilli and of erythrocytes sensitized with the components of pertussis bacilli. In this labortory, Kinoshita *et al.*[19] demonstrated a similar specific hemolytic reaction of bovine erythrocytes sensitized with components of typhoid bacilli by adding the antityphoid immun serum with complement. Under the guidance of Prof. Kuroya and Prof. Nakamura, we sensitized bovine erythrocytes with Dorset-Tuberculin and tested upon the sera of tuberculous patients in the same manner. Healthy guinea-big serum was used as the complement. Some



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conditions to perform the reaction were examined at first and then the procedure was applied to 100 tuberculous patients and 90 non-tuberculous, practically healthy adults. Further the specificity of this reaction and its relation with clinical symptoms were discussed. Meanwhile, in a literature obtained recently, we found that Fisher and Keogh[3] and Middlebrook[20] also conducted this hemolytic reaction by adding complement to their reaction system. However, no comparable study with clinical findings were performed. In these respects, it will be noteworthy to present the results obtained in this paper.

## Experimental

### Material and Method

Bovine erythrocytes were used for the test. Namely the defibrinated blood was washed repeatedly with physiologic saline and after the centrifugation at 2,000 r.p.m. for 3 minutes, original stock cells were prepared.

The sensitized erythrocytes suspension was prepared in the following manner. Dorset-Tuberculin given from Research Institute for Tuberculosis and Leprosy, Tohoku University, was diluted to 1:10 with physiological saline and adjusted to pH 7.0. It was added with 1/10 volume of 10 per cent blood suspension, mixed sufficiently and incubated at 37°C for 2 hours with occasional shaking. After the centrifugation, the supernatant was discarded and further sensitized cells were washed 3 times with physiological saline in order to remove the excess of tuberculin. The lengths of time and the density of tuberculin required to sensitize the erythrocytes were tested experimentally and shown in Table I and II. According to the results shown in the Table we decided to sensitize the cells for 2 hours with 1/10 diluted tuberculin.

The sera of patients were inactivated by heating at 56°C, for 30 min., mixed thoroughly with the same volume of original stock bovine

Table I

Time Required to Sensitize the Erythrocytes

| Time of sensitizing | Serum dilution | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1:3 | 1:6 | 1:12 | 1:24 | 1:48 | 1:96 | 1:192 |
| 30 min. | + | + | + | ± | − | − | − |
| 60 " | + | + | + | + | − | − | − |
| 90 " | + | + | + | + | ± | − | − |
| 120 " | + | + | + | + | + | − | − |
| 150 " | + | + | + | + | + | − | − |

Complement used, 0.25 cc. of 1:10 diluted guinea pig serum.

TABLE II

The Density of Tuberculin Required to Sensitize the Erythrocytes

| Tuberculin | Serum dilution | | | | |
|---|---|---|---|---|---|
| | 1:10 | 1:20 | 1:40 | 1:80 | 1:160 |
| 1:10 | + | + | + | − | − |
| 1:100 | + | − | − | − | − |
| 1:1000 | − | − | − | − | − |

Complement used, 0.25 cc. of 1:10 diluted guinea pig serum.

cells and allowed to stand for 1 hour at 0°C to absorb the normal hemolysin. The serums drawn from 5–6 guinea pigs were mixed to maintain the constant titer and the normal hemolysin was removed in a similar way as described above. Finally it was diluted to 1:10 with physiological saline and used as complement. The serum of patients was diluted in two fold serially starting from the dilution 1:3 and used in amount of 0.5 cc. as shown in Table III. To the diluted sera were added 0.5 cc. of sensitized cells and 0.25 cc. of complement and mixed sufficiently and the mixture was kept at 37°C. After 2 hours, the definite titration of the serum was read by the degrees of hemolysis. The final dilution re-

TABLE III

Procedures for Main Test

| Assay | | 0.1% sensitized red cells 0.5 cc. | Patient's serum 0.5 cc. | 1:10 complement 0.25 cc. | Physiological saline 0 |
|---|---|---|---|---|---|
| Control | 1 | " 0.5 cc. | 0 | 0 | " 0.75 cc. |
| | 2 | " 0.5 cc. | " 0.5 cc. | 0 | " 0.25 cc. |
| | 3 | " 0.5 cc. | 0 | " 0.25 cc. | " 0.5 cc. |
| | 4 | 0.5% non-sensitized red cells 0.5 cc. | " 0.5 cc. | " 0 | " 0.25 cc. |
| | 5 | " 0.5 cc. | 0 | " 0.25 cc. | " 0.5 cc. |
| | 6 | " 0.5 cc. | 0 | 0 | " 0.75 cc. |
| | 7 | " 0.5 cc. | " 0.5 cc. | " 0.25 cc. | 0 |
| Positive control | | 0.5% sensitized red cells 0.5 cc. | Positive serum 0.5 cc. | " 0.25 cc. | 0 |
| Negative control | | " 0.5 cc. | Negative serum 0.5 cc. | " 0.25 cc. | 0 |

quired to cause a complete hemolysis was designated as a serum titer. We found as described later, that the rejection limit of serum dilution of healthy subjects having negative tuberculin test is 1:2.054. Therefore, we determined the test as positive when the hemolytic reactions occurred in lower than 1:3 dilution, and as negative even if it was positive in higher than 1:2 dilution.

As the control 9 sets of test shown in Table III were conducted and the ordinary test was read under the condition that these control tests were carried out satisfactorily.

## Results

The reaction was applied to 100 cases of tuberculous patients and 90 cases of control including non-tuberculous patients and healthy adults. The results were summarized in Fig. 1. The significant differences were

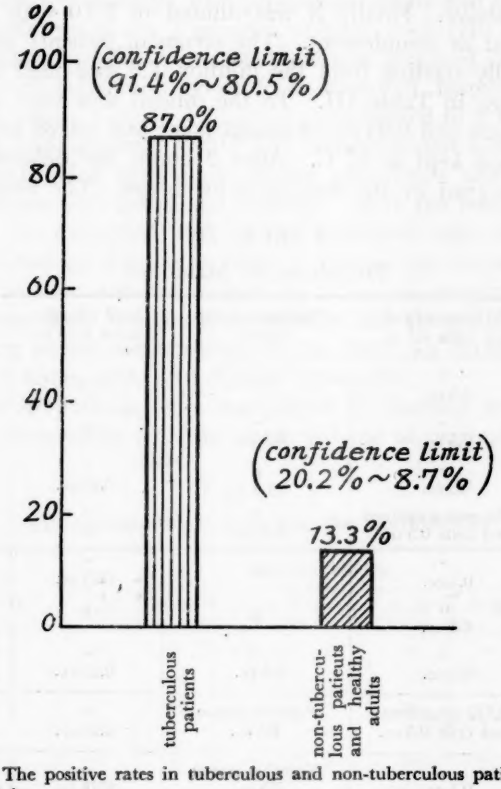


Fig. 1. The positive rates in tuberculous and non-tuberculous patients and healthy adults.

proved statistically between the tuberculous patients showing the positive rate 87.0 per cent (confidence limit 91.4–80.5 per cent), and non-tuberculous patients showing that of 13.2 per cent (confidence limit 20.2–8.7 per cent). Also as shown in Table IV, almost all tuberculous patients showing the positive reaction possessed the high titer such as 1:12 to 1:96. Subsequently, the following correlation between the clinical symptoms and the titer were recognized.

*The relation with the stages of disease (based on Turban-Gerhardt's classification).* The differences of titer persued according to the stages of disease were scarcely recognized, except that a somewhat higher positive rate was found in 3rd stage as shown in Table V and a high titer such as 1:48 was observed in many cases of 2nd and 3rd stage.

TABLE IV

The Positive Rates in Tuberculous Patients

| | Negative | Number of positive cases in serum diluted | | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1:3 | 1:6 | 1:12 | 1:24 | 1:48 | 1:96 | 1:192 | 1:384 | |
| Cases | 13 | 8 | 6 | 16 | 14 | 23 | 13 | 6 | 1 | 100 |
| Rates | | 8% | 6% | 16% | 14% | 23% | 13% | 6% | 1% | 87.0% |

TABLE V

The Relation with the Stages of Disease (based on Turban-Gerhardt's classification)

| Stage of disease | | I | II | III |
|---|---|---|---|---|
| Negative | | 1 | 8 | 3 |
| Positive with the serum diluted | 1:3 | 0 | 5 | 0 |
| | 1:6 | 1 | 2 | 2 |
| | 1:12 | 0 | 9 | 5 |
| | 1:24 | 3 | 6 | 3 |
| | 1:48 | 0 | 13 | 10 |
| | 1:96 | 1 | 5 | 5 |
| | 1:192 | 0 | 3 | 2 |
| | 1:384 | 0 | 0 | 1 |
| Total | | 6 | 51 | 31 |
| Positive cases | | 5 | 43 | 28 |
| Positive rate | | 83.3% | 84.3% | 90.3% |

TABLE VI

The Relation with the Forms of Disease

| Forms of disease | | Mixed form | Productive form | Exsudative form |
|---|---|---|---|---|
| Negative | | 1 | 9 | 2 |
| Positive with the serum diluted | 1:3 | 4 | 1 | 1 |
| | 1:6 | 1 | 4 | 0 |
| | 1:12 | 7 | 7 | 1 |
| | 1:24 | 3 | 8 | 2 |
| | 1:48 | 12 | 8 | 0 |
| | 1:96 | 8 | 3 | 0 |
| | 1:192 | 4 | 1 | 0 |
| | 1:384 | 1 | 0 | 0 |
| Total | | 41 | 41 | 6 |
| Positive cases | | 40 | 32 | 4 |
| Positive rate | | 97.6% | 78.0% | 66.7% |

*The relation with the forms of disease.* 88 cases of pulmonary tuberculosis were classificated into 3 forms, i.e., exsudative, productive and mixed form.

The mixed form showed the highest positive rate of 97.6 per cent, the productive form showed a slightly lower and the exsudative form the lowest. In the mixed form, several cases showed a high titer from 1:48 to 1:96, while in the productive form almost all sera of patients showed a low titer from 1:12 to 1:48. Namely it was found that the mixed form showed a higher hemolytic titer than the productive form.

*The relation with tuberculin test.* As shown in Table VII, there is a tendency to be shown a higher positive rate in cases which were strongly positive in tuberculin test. However, the correlation between the degree of tuberculin test and hemolytic titer was not able to be ascertained. Previously Yaoi *et al.*[14] also described that the hemagglutination reaction did not progress in parallel with the tuberculin test.

*The relation with blood sedimentation rate.* As shown in Table VIII, a relatively high positive rate of serum was shown in case which had a moderately high blood sedimentation rate. Subsequently, it was found that most parts of patients showed the hemolytic titer of 1:48 irrespective of blood sedimentation rate. Consequently, it was proved, that no particular relation between blood sedimentation rate and this test was able to be found.

TABLE VII

The Relation with the Tuberculin Test

| Tuberculin test (diameter of redness) | | 10–19 mm. | 20–39 mm. | over 40 mm. |
|---|---|---|---|---|
| Negative | | 2 | 9 | 2 |
| Positive with the serum diluted | 1:3 | 0 | 7 | 1 |
| | 1:6 | 3 | 1 | 2 |
| | 1:12 | 1 | 13 | 2 |
| | 1:24 | 1 | 7 | 6 |
| | 1:48 | 2 | 16 | 5 |
| | 1:96 | 1 | 10 | 2 |
| | 1:192 | 1 | 3 | 2 |
| | 1:384 | 0 | 1 | 0 |
| Total | | 11 | 67 | 22 |
| Positive cases | | 9 | 58 | 20 |
| Positive rate | | 81.8% | 86.5% | 90.0% |

TABLE VIII

The Relation with the Blood Sedimentation Rate

| Blood sedimentation rate (mm.) | | 1–19 | 20–39 | over 40 |
|---|---|---|---|---|
| Negative | | 10 | 1 | 2 |
| Positive with the serum diluted | 1:3 | 7 | 0 | 2 |
| | 1:6 | 4 | 2 | 0 |
| | 1:12 | 11 | 1 | 3 |
| | 1:24 | 12 | 2 | 0 |
| | 1:48 | 15 | 5 | 3 |
| | 1:96 | 10 | 1 | 2 |
| | 1:192 | 4 | 2 | 0 |
| | 1:384 | 1 | 0 | 0 |
| Total | | 74 | 14 | 12 |
| Positive cases | | 64 | 13 | 10 |
| Positive rate | | 86.5% | 92.9% | 83.3% |

*The relation with the expectoration of tubercle bacilli in sputum.* As shown in Table IX, the patients having negative evidence of tubercle bacilli both by smear and cultivation of sputum showed a higher positive rate than the patients having positive evidence. All of 17 cases which are now showing negative evidence of bacilli, but formerly when they entered to the clinic had positive evidence, proved positive results.

TABLE IX

The Relation with the Expectoration of Tubercle Bacilli in Sputum

| Tubercle bacilli in sputum | | (−)→(−) | (+)→(−) | (+)→(+) | (−)→(+) |
|---|---|---|---|---|---|
| Negative | | 3 | 0 | 9 | 1 |
| Positive with the serum dilution | 1:3 | 4 | 1 | 3 | 0 |
| | 1:6 | 2 | 3 | 1 | 0 |
| | 1:12 | 3 | 5 | 8 | 0 |
| | 1:24 | 4 | 0 | 10 | 0 |
| | 1:48 | 7 | 3 | 14 | 0 |
| | 1:96 | 2 | 4 | 6 | 0 |
| | 1:192 | 1 | 1 | 4 | 0 |
| | 1:384 | 0 | 0 | 1 | 0 |
| Total | | 26 | 17 | 56 | 1 |
| | | 43 | | 57 | |
| Positive cases | | 23 | 17 | 47 | 0 |
| | | 40 | | 47 | |
| Positive rate | | 88.5% | 100.0% | 83.9% | |
| | | 93.0% | | 83.9% | |

On the other hand, the test was employed to 22 cases of nontuberculous patients. 18 cases of them including 3 cases of syphilis showed negative reaction (81.8 per cent). The remaining 4 cases gave a positive reaction, which are containing 2 cases of diabetes mellitus, 1 case of rheumatism and 1 case of nephritis. 17 of 21 cases, who were practically healthy with a tuberculous anamnesis, showed a negative reaction as in dicated in Fig. 2. Among these 21 cases, 6 had no particular change in the chest on the X-ray examination, 12 had the cirrhotic change (4 who showed the positive reaction were included in this group), and 2 had the pleural adhesion or pleural induration.

Among the 50 cases which showed positive tuberculin test without

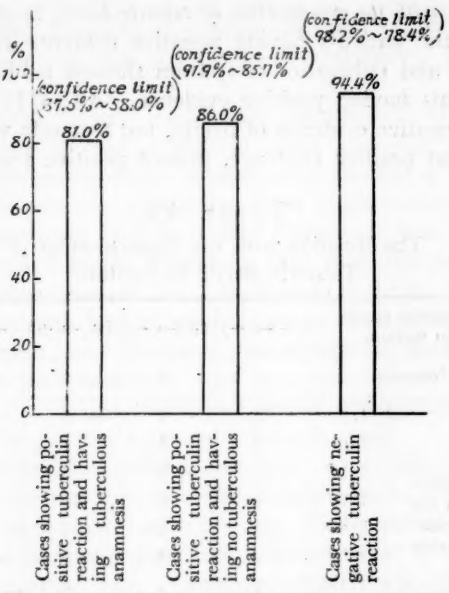


Fig. 2. Negative rates in nontuberculous patients and healthy adults.

the tuberculous anamnesis, 43 cases showed negative reaction and 7 cases showed positive reaction, and 5 of latter 7 cases were considered in X ray photograph that they had the tuberculous anamnesis unconsciously in past, and all of these 7 cases showed the low titer of less than 1:24. Among 18 cases having negative tuberculin test, 94.4 per cent showed the negative reaction, and the only one case who showed the positive reaction possessed the titer of 1:3 only. It was considered to be interesting, that the latter case who showed the positive reaction had the positive tuberculin reaction once after the B. C. G. inoculation and then returned to negative again.

## Discussion

1) The hemolytic reaction described here is ultimately a kind of the complement fixation test. The complement fixation test was the only one which hitherto used to prove the antibody in the sera of tuberculous patients, and also this test will be employed similarly to prove the specific antibody. However, this hemolytic test seems to have the significant differences in the theoretical meaning and seems to be more convenient for its easy procedure and for its specifity for active tuberculosis. Previously, Kimura[21] reported that a high positive rate and a high hemolytic titer were shown in 3rd stage of tuberculosis, utilizing

the complement fixation test. The similar results were also found in this test. Kusunoki[22] and Kimura[21] showed that the complement fixation test advanced in parallel with blood sedimentation rate, although the same fact was not proved in this hemolytic reaction.

2) This test seems to be more insensitive than the tuberculin reaction in a simple comparison. But, in diagnostic meaning, a low positive rate and a low hemolytic titer were found in the cases who were working in their own job, even if they had the tuberculous anamnesis in their past days. Contrary to this, in the cases who had active tuberculosis, a high positive rate and a high hemolytic titer were found. Therefore, we consider that the reaction would be useful for diagnosis of active tuberculosis.

3) When this hemolytic test is compared with Middlebrook's hemagglutination test, the former seems to have more advantages. Concerning the positive and negative percentage of tuberculous and nontuberculous adults, there might be no recognizable differences, but in the former reaction the results will be decided more easily and decidedly in a shorter time, and in a more precise manner. Further, when compared these 2 tests in its specificity, hemolytic reaction seems to be more specific. That is concerning with the titer of leprosy serum tested with tuberculin sensitized cells. In the hemagglutination test, Levine[23] proved, that 90 per cent was positive among 80 cases of tubercular leprosy and that the serum titer was as high as 1:8 to 1:2048, whereas 44 per cent of 24 cases of nervous leprosy were positive showing the titer of 1:8 to 1:128. It must be emphasized, that the hemagglutination titer and the positive rate obtained in these cases are higher than that of tuberculosis. Mayama and Fukuda[24] also showed the approximately the same results in the hemagglutination test using chicken red cells. However, Aono[25] who employed the similar hemolytic method in this laboratory described that in 25 cases of lepromatous type only 32 per cent showed positive result, among which the highest titer was 1:100, while in 11 cases of neuromacular type only one case gave positive reaction showing the hemolytic titer of 1:100. Namely the hemolytic titer and the positive rate in leprosy cases were both lower than those obtained in tuberculous patients, contrary to the hemagglutination test.

4) Kinoshita *et al.*[19] reported that in their experiments on the sensitization by the components of *S. typhi*, the bovine erythrocytes were the best for the hemolytic examination. Therefore, bovine erythrocytes were used in this experiment too. On the other hand Yaoi *et al.* described that chicken red cells were the most suitable in their hemagglutination test. In 7 cases of hemagglutination tests with bovine erythrocytes which were done simulataneously with hemolytic test, it was proved that, the former was all negative, while the latter all positive. According to these facts, it

seems that bovine erythrocytes are suitable for hemolytic reaction, whereas chicken red cells are better in the hemagglutination test.

5) It will be anticipated that an antigen contained in tuberculin, a component of body substance of tubercle bacilli, may produce an antibody in tuberculous patients, and by the hemolytic test using red blood cells sensitized with tuberculin, it may be possible to detect the distribution of antibody not only in tuberculous patients but also in apparently "non-tuberculous" peoples. These examinations were persued, and when the population number was taken on ordinate and the serum dilution expressed in logarithmic number on abscissa, Gauss' normal distribution curve was obtained. The statistical analysis indicated the population mean of 1.06 and rejection limits of 2.054 (with 5 per cent of level of significance). According to the results hemolytic titer above 3 was decided as positive as mentioned above.

## Summary

1. The hemolytic reaction, using bovine erythrocytes sensitized with Dorset tuberculin, was examined in the sera of tuberculous and non-tuberculous peoples and the ordinary procedure was established.

2. Among the tested, 87 of 100 cases of tuberculous patients showed the positive results, while 13 of 90 cases of nontuberculous adults also gave a positive reaction. Among 90 non-tuberculous controls, 4 of 22 cases having no history of tuberculous, 4 of 21 cases showing the positive tuberculin test and having tuberculous anamnesis in their past, 7 of 50 cases having the positive tuberculin test without tuberculous anamnesis, and 1 in 19 cases having negative tuberculin test showed a positive reaction. The hemolytic titers of such a non-tuberculous adults were much lower when compared with those of tuberculous patients.

3. The relations between the degree of the hemolytic reaction and clinical symptoms, namely the stage of disease, the form of disease, the tuberculin reaction, the blood sedimentation rate and the number of tubercle bacilli in sputum, were discussed on 100 cases of tuberculous patients.

4. The hemolytic reaction is a specific one for tuberculosis, and seems to be so useful as a supplementary diagnostic method of active tuberculosis.

The authors wish to express their hearty thanks to Prof. M. Kuroya and to Prof. T. Nakamura for their helpful advices and for their kind criticism.

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# Interconversion of Blood Group Lipoids A and B *in vitro* and N-Glycoside Linkage in Group Substances A and B in General*

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It was found that the Group B lipoid of hog lung[1] changes in part into a Group A substance (determined by iso-agglutination inhibition test) on standing in physiological saline. The optimum pH was 7.0, just as for the $\alpha$, $\beta$-rearrangement of N-glycosides. The change, which was hardly noticed at 0°C, occurred more and more markedly with raising the temperature of the solution, and when the latter was again cooled to 0°C, the A activity diminished gradually. That the appearance of A activity is not due to formation of a non-specific anti-agglutinative agent was confirmed by an absorption experiment by the use of anti-A and anti-B agglutinin. Interconversion of Group lipoids A and B of human liver could be caused by procedures different from above.

We subjected those group lipoids to partial alkaline cleavage so as to separate a group-potent portion rich in carbohydrate. A spot on a filter-paper of the scission products, placed after dissolving in a buffer of pH 5.0 and maintaining at 50°C for 5 hrs. (in moistened room), gave a more vivid ninhydrin reaction than the control spot without the prior warming of the solution. They also gave positive result in retention analysis with azocarmine, when heated in advance to 40° with a dilute acetic acid of pH 2.0. On the other hand, they did not reduce a hot mercuric chloride solution at pH's from 7.5 to 8.0, but were Fehling-positive. In these regards, the group carbohydrate (amino acid-polysaccharide complex) from hog stomach mucus[2] proved to resemble them.

Those consequences of chemical tests indicate that the carbohydrate in the group substances combines with the polypeptide residue at its reducing terminal through N-glycoside linkage. We[3] have already informed that a Group A carbohydrate from an animal tissue or fluid has positive rotation (optical) or less negative rotation than the corresponding Group B carbohydrate from the same source in spite of its identical analytical composition (such group carbohydrates as those from long-stagnating cyst fluids excluded), if the factors are isolated in one and the same manner. Thus it may be most plausibly assumed that Group A and B substances are $\alpha$- and $\beta$-N-glycosides respectively.

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* Read before the IInd General Meeting of the Japanese Union of Physiological Sciences, Tokyo, November 10, 1952.

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# A Hemolytic Reaction of Erythrocytes Sensitized With Tuberculin and Components Extracted from Leprosy Nodules

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In recent years, Middlebrook and Dubos[1] has published a hemagglutination reaction of erythrocytes sensitized with extracts of tubercle bacilli and it was retested by many investigators. Subsequently the test was made in leprosy by Levine[2] with sheep red cells sensitized with tuberculin. It was revealed that the test was applicable for measuring a serological responce in leprosy patients and that antibody titers in active cases were significantly higher than those in negative cases and frequently much higher than those encountered in active cases of tuberculosis and the reason of which was thought to be due to the fact that *M. leprae* and *M. tuberculosis* possess a common antigenic structure. As compared with this work, Koji[3] made a same experiment and reported on the lower titer in leprosy than in tuberculosis.

Recently, Mayama and Fukuda[4] made a test with hen's erythrocytes and concluded that the titers in lepromatous type were higher than those in neuro-macular type, and that the more advanced the clinical conditions of patients, the higher the titer of sera.

We[5] have made a fundamental experiment on a hemolytic reaction between the cattle erythrocytes sensitized by body components of typhoid bacilli and anti-typhoid sera with addition of complement, and found that the hemolytic reaction was more sensitive and specific than the other hitherto known serological reactions, and the author attemped to apply this reaction for leprosy. Because the artificial cultivation of *M. leprae* is not yet successful, instead of extracts of this bacilli that from leprosy nodules must be used. In connexion with this, were reported formerly some detailed investigations by Nakamura[6] in our laboratory and Toda.[7] But it is very difficult to gather a large quantity of nodules on account of the recent developement of chemotherapy in leprosy. Thereby, on the other hand is described here a hemolytic reaction of erythrocytes

sensitized with tuberculin as reported by Levine, in order to know whether tuberculin can substitute for extracts of *M. leprae* or not.

## Experimental

### Materials and Methods

1) Experiment using erythrocytes sensitized with tuberculin

The technic employed for determination of titers of hemolytic reaction is that described by our co-workers.[8]

2) Experiment using erythrocytes sensitized with extracts from leprosy nodules

Leprosy nodules 5.2 g. (wet weight) are immediately cut into thin slices and then placed in five volumes of a mixture of 96 per cent. alcohol and ether (1:1 by volume). After a lapse of one week, the mixture was centrifuged and the residue was then treated with about ten volumes of boiling alcohol for 17 hours, and then the mixture was centrifuged again and the residue dried in a desiccator. It was then treated with boiling water five times (1.5 hours) and the supernatant was dialyzed. The water extract was concentrated under reduced pressure, the appearing precipitates centrifuged off.

To the yellowish supernatant liquid were added equal volume of 1/2 *N* trichloroacetic acid and the flocculent precipitates centrifuged. The supernatant was dialyzed and added with three volumes of acetone and the appearing precipitates was dried in vacuo (CF).

The precipitates produced by trichloroacetic acid were redissolved again in water with addition of small amount of alkali and precipitated in its IEP and dried using lyophilisation ($PF_1$).

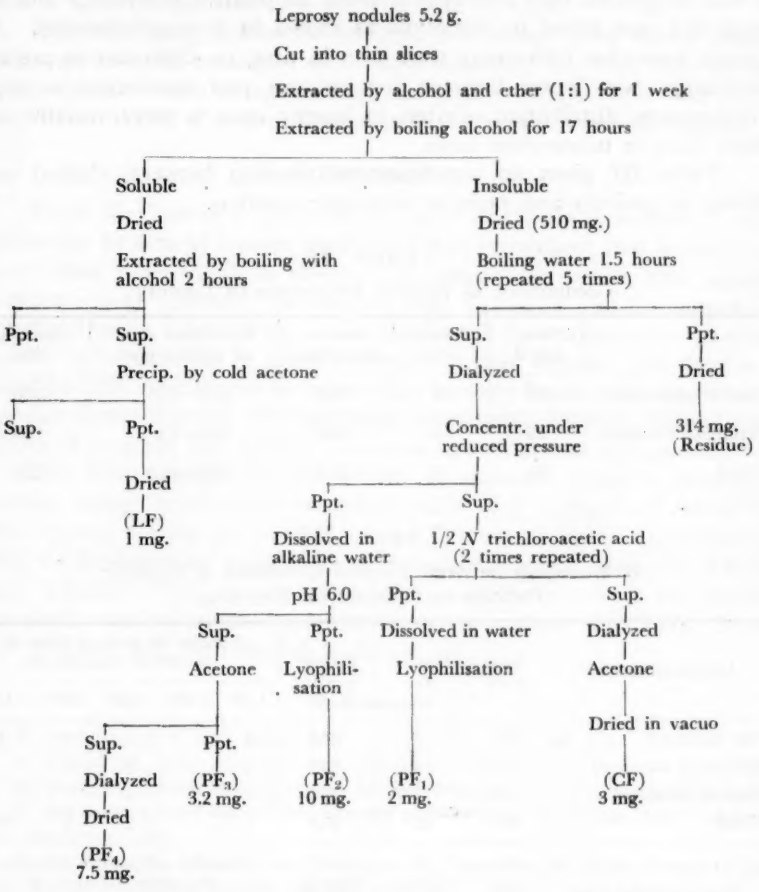
The precipitates appearing during concentration of the original water extract under reduced pressure gave positive biuret and ninhydrin tests and were dissolved in water in alkaline condition and precipitated at IEP (pH 6.0). The precipitates were dried by lyophilisation ($PF_2$). The supernatant was again precipitated with acetone and dried ($PF_3$). The last supernatant was dialyzed and dried ($PF_4$).

The cold and hot alcohol extracts were evaporated together under reduced pressure to dryness, and the remainder again treated with boiling alcohol. To the hot alcohol soluble part were added five volumes of cold acetone and the appearing white precipitates were dried (LF). The outline of these procedures and yields of each fraction were showed in Table I.

Approximately 1 cc of washed 10 per cent red cells was added to 2 cc. of the neutral isotonic solutions containing 1 mg. of each of these fractions. The cells were well suspended and placed in a water bath

TABLE I

Separation of Several Components from Leprosy Nodules




at 37°C, for 2 hours being frequently agitated, then the suspension was centrifuged and the clear supernatant fluid was discarded. The packed treated red cells were washed three times with 30 cc. of saline and finally suspended in 20 cc. of saline (0.5 per cent "sensitized red cell suspension").

## Results

*Experiment using erythrocytes sensitized with tuberculin.* As shown in Table II, 12 sera of the 40 lepromatous type and 5 sera of the 25 neuro-

macular type showed positive hemolysis in dilution of 10 to 100 times of sera. Namely, in contrast with tuberculous cases by Takasawa *et al.* it was recognized that leprosy was lower in positive percentage and the same fact was found in hemolysis as shown in hemagglutination. Although somewhat differences were seen in titer, no difference in positive percentage was found between lepromatous and neuromacular type. Furthermore, distribution of titers in leprosy cases is more variable and labile than in tuberculous cases.

Table III gives no significant relationship between clinical conditions of patients and positive hemolytic reaction.

TABLE II

Comparison of Positive Percentage in Leprosy

| | Sera tested | Positive sera | Positive percentage | Confidence limit of positive perc. | Averaged titer |
|---|---|---|---|---|---|
| Lepromatous type | 40 | 12 | 33.3 | 50.2–12.3 | 2.68 |
| Neuromacular type | 25 | 5 | 20.0 | 42.6–4.2 | 1.99 |
| Total | 65 | 17 | 26.1 | 40.8–13.5 | 2.38 |

TABLE III

Relationship between Clinical Condition of Leprosy Patients and Hemolytic Reaction

| Lepromatous type | Sera tested | Positive sera | Positive percent. | Number of positive cases at the serum dilution of | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | 1:10 | 1:20 | 1:40 | 1:80 | 1:100 |
| Far advanced | 17 | 7 | 41.1 | 4 | | | | 3 |
| Mediately advanced | 18 | 4 | 27.7 | 2 | | 1 | 1 | |
| Slightly advanced | 5 | 1 | 20.0 | | | 1 | | |
| Total | 40 | 12 | 33.3 | 6 | | 2 | 1 | 3 |
| **Neuro-macular type** | **Sera tested** | **Positive sera** | **Positive percent.** | **Number of positive cases at the serum dilution of** | | | | |
| | | | | 1:10 | 1:20 | 1:40 | 1:80 | 1:100 |
| Far advanced | 4 | 1 | 25.0 | | | 1 | | |
| Mediately advanced | 13 | 2 | 15.3 | | | 1 | | 1 |
| Slightly advanced | 8 | 2 | 25.0 | 1 | 1 | | | |
| Total | 25 | 5 | 20.0 | 1 | 1 | 2 | | 1 |

In addition to this experiment, a hemolytic reaction was conducted between the erythrocytes sensitized with a polysaccharide fraction of

typhoid bacilli (Ashida[9]), alkali treated Boivin antigen) and the sera of leprosy patients. Consequently 2 cases of 8 lepromatous type and 1 case of 6 neuro-macular type showed titer of 1:10.

*Experiment using erythrocytes sensitized with extracts from leprosy nodules.* Using erythrocytes sensitized with above described fractions from leprosy nodules, each 5 sera of lepromatous and neuro-macular type were tested in 1:10 to 1:100 of serum dilution and the results were all negative.

## Summary

From the hemolytic reaction of cattle erythrocytes sensitized with tuberculin by sera of leprosy patients, it was recognized that leprosy was lower than tuberculosis in positive percentage and serum titer against Levine's report. Namely, in contrast with Takasawa and his coworker's investigations in tuberculosis, it was confirmed that tuberculin was more specific to tuberculosis in hemolytic reaction than leprosy, and there was found no difference of positive percentage between lepromatous and neuro-macular type. Further, the significant relationship between clinical conditions of patients and positive hemolytic reactions was not proved.

The author could not also obtain an expected result in hemolytic reaction using erythrocytes sensitized with each component extracted from leprosy nodules because of a small amount of yields. Considering from the fundamental experiment of hemolytic reaction, however, if more large quantity of the extract of nodules would be obtained, the specific hemolytic reaction would appear in leprosy as in cases of typhoid. Now, this problem will be revealed in future.

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# Experimental Tuberculous Infection in Mice

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Since many successes have followed in the discovery of antibiotics curative in acute bacterial infections, our special attention has been focused on those diseases, against which such success has not yet been so satisfactory, especially on human tuberculosis.

As reported already in our systematic studies on actinomyces anti biotics, roseomycin, anti-phlei factor (No. 364), flaveolin, glyseoflavin and vinacetin have been isolated and they have shown some activities against tubercle bacilli in vitro.

But, a further study required the establishment of standardized animal experiment,[1)2)3)4)] i.e. the selection of a satisfactory experimental animal and a simple quantitative pursuance method of the bacilli in the tuberculous tissues.

Since the discovery of streptomycin, animal experiments against *Mycobacterium tuberculosis* were discussed by many authors.[5)6)7)] Some of them used guinea-pigs, and others used mice. Although in the past the rabbit and mouse have also been used, most of the authors chose guinea-pigs for chemotherapeutic experiments.

In my opinion, however, it is a so interesting fact, that the intravenous inoculation of the bacilli into mice causes a specially severe lesion of the lung as shown in human beings.

In this respect Arimura[1)] *et al.* discussed the specificity of mice tuberculosis comparing with that of other animals, and Youmans[2)] emphasized the characteristic lung lesion in the curative experiment by streptomycin.

Recently McKee *et al.*[5)] described many advantages of mice tuberculosis using the pure bleed strains. The use of mice in chemotherapeutic experiment against tuberculosis will present the following advantages:

1) The estimation of the effectiveness will be decided in a rather short time.

2) A relatively small quantity of the antibiotic will be suffcient to treat the infected animals, and as the results, we can treat many animals with small quantities of remedies. From the economical point too, it

enabled us to use the large number of animals in such an experiment.

3) As described above, a special lesion in lungs is a favourable fact, for the final purpose is chiefly concerned with the human lung tuberculosis.

4) The recent advances in the quantitative estimation of tubercle bacilli in tissues will afford the statistical treatment of the results.

5) Even the cross breed mice have shown identical changes, being compared with the pure breed ones, as shown in my preliminary experiments. So, when summarized results of the experiment will be judged from these points of view, not only from pathological changes, the use of mice has no shortage being compared with that of guinea-pigs.

From the above point of view, some experiments of mice tuberculosis were conducted and will be reported here.

## Experimental

Exp. I. The distribution of living tubercle bacilli in several viscera after intraperitoneal injection of avian tubercle bacilli, " Flemingo " strain.

Twelve white mice (mixed breed), weighing 15 to 18 g., were divided into 4 groups, composed of 3 animals each, and infected intraperitoneally with 0.1, 0.25, 0.5 and 1.0 mg. (moist weight) of Flemingo strain, respectively. At 10th, 20th and 30th day after inoculation one animal of each group was sacrificed and tested upon the amount of bacilli from lung, liver, spleen, mesenterial lymph node and ascitic fluid. The quantitative cultivation method was conducted according to Ogawa[10][11] as follows. Each of the organs was removed, weighed, and the weighed amounts of them were completely ground in a porcelain mortar, adding 10 volumes of dil. sulphuric acid (4 per cent). After centrifugation 3000 r.p.m. for 10 min., the residue was suspended in the same volume of physiological saline, diluted further in 10 fold serially and 0.2 cc. of these suspensions were inoculated into each of 3 tubes of Oka-Katakura's egg media. After cultivation at 37°C for 4 weeks, the number of colonies were counted.

As shown in Table I,

1) 2 mice died in the course of the experiment, and the reason of death was confirmed to be not of tuberculosis by necropsic findings.

2) Macroscopically, the extensive involvement of the lung was never seen in any animals. Only in severe infected groups (0.5 and 1.0 mg. each), slight pathological changes due to tuberculosis were found in the spleen, liver and partially in lymph node.

3) The tubercle bacilli were already proved after 10 days from the infection, especially in the liver, spleen and lymph nodes, but even after 30 days the number of colonies was not so increased. It is of interest, that there is no dissemination of the bacilli in lung by the intraperitoneal

TABLE I

The Distribution of Living Tubercle Bacilli in Various Viscera, after Intraperitoneal Injection of Avian Tubercle Bacilli (Flemingo Strain)

| Organs | | | Lung | | Liver | | Spleen | | Lymph nodes | | Ascitic fluid | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Group (inoculated dosage in mg.) | After inoculation (days) | Result | Number of colonies* | Pathological changes† | Number of colonies | Pathological changes‡ | Number of colonies | Pathological changes† | Number of colonies | Pathological changes† | Number of colonies | Pathological changes |
| I (0.1) | 10 | killed | − | − | ## | | ++ | − | ++ | − | + | − |
| | 14 | died | − | − | ++ | | ++ | − | ++ | − | + | − |
| | 30 | killed | − | − | ++ | | ++ | + | ++ | − | + | − |
| II (0.2) | 8 | died | ++ | − | ++ | | ++ | − | ## | − | − | − |
| | 10 | killed | ++ | − | ## | | ++ | − | ## | − | + | − |
| | 30 | killed | + | − | ## | | ## | − | ## | − | + | − |
| III (0.5) | 10 | killed | + | − | ## | | ++ | + | ++ | − | ++ | − |
| | 20 | " | ++ | − | ## | | ## | ++ | ++ | − | + | − |
| | 30 | " | + | − | ## | | ## | ++ | ++ | − | ++ | − |
| IV (1.0) | 10 | killed | + | − | ++ | | ++ | ++ | ++ | − | + | − |
| | 20 | " | ++ | − | ## | | ## | ++ | ## | − | + | − |
| | 30 | " | ++ | − | ## | | ## | ++ | ## | ++ | + | − |

* Number of colonies was indicated by the symbol −, +, ++ and ##, according to the degrees of the bacterial growth.

† The graded phathological changes were judged as follows:

| Lung: | Spleen (expressed in weight): | Mesenterial lymph nodes: |
|---|---|---|
| − No tubercle | | |
| + Dissemination of smallest tubercles (0.5–1.0 mm. in diameter) | − Below 100 mg. | − No tubercle |
| | + Over 100 „ | + Small tubercle |
| ++ Dissemination of small tubercles (1–2 mm. in diameter) | ++ Over 200 „ | ++ Middle „ |
| | ## Over 300 „ | ## Large „ |
| ## Many tubercles | ### Over 400 „ | |
| ### Many large tubercles were adhered with each other | | |

‡ Macroscopic findings of the liver were difficult to be recorded in score, so data were not shown.

infection especially in the case of small inoculum (0.1 mg.).

Exp. II. The distribution of living bacilli in several viscera after

intravenous infection of human tubercle bacilli ($H_{37}Rv$).

27 white mice weighing 14–18 g. were divided into 3 groups composed of 9 mice each. Each of 3 groups was inoculated intravenously with 0.01, 0.02 and 0.05 mg. (moist weight) of virulent human tubercle bacilli ($H_{37}Rv$), respectively. At 15th, 25th and 35th day after infection, 2–3 mice of each group were killed and tested on the number of bacilli from lung, liver, spleen, lymph nodes and ascitic fluid.

The applied method here is as follows. For the first autopsy,[9] after 15 days the weighed amount of each of organs was ground in a porcelain mortar with 10 volumes of sulphuric acid (4 per cent) under aseptic condition, and the suspension was centrifugated 3000 r.p.m. for 10 min. The supernatant was discarded, the residue was resuspended in the same volume of physiological saline and diluted in 10 fold serially. 0.5 cc. of the suspension were inoculated into each of 3 tubes of Oka-Katakura's egg media. In 2nd and 3rd autopsy, however, the weighed amount of each of organs was ground in 10 volumes of 4 per cent NaOH solution, diluted in 10 fold serially and 0.2 cc. of the suspensions were inoculated

TABLE II

The Distribution of Living Tubercle Bacilli in Various Viscera, after Intravenous Injection of Human Tubercle Bacilli ($H_{37}Rv$ Strain)

| Group (inoculated dosage in mg.) | After the inoculation (days) | Number of mice tested | Organs | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Lung | | Liver | | Spleen | | Lymph nodes | | Ascitic fluid | |
| | | | Number of colonies* | Pathological changes | Number of colonies | Pathological changes† | Number of colonies | Pathological changes | Number of colonies | Pathological changes | Number of colonies | Pathological changes |
| I (0.01) | 15 | 3 | 0.3 | − − − | 0.3 | | 0.7 | − − + | 0 | − − − | 0 | − − − |
| | 25 | 3 | 6.4 | − + + | 0.6 | | 6.0 | + + + | 0 | − − − | 0 | − − − |
| | 35 | 2 | 21.0 | + ++ | 1.1 | | 15.5 | ++ ++ | 0.03 | − − | 0 | − − |
| II (0.02) | 15 | 2 | 1.0 | − − | 0.3 | | 2.0 | ++ | 0 | − − | 0 | − − |
| | 25 | 3 | 11.8 | + ++ + | 0.9 | | 9.1 | ++ ++ ++ | 0 | − − − | 0 | − − − |
| | 35 | 3 | 18.0 | ++ + ++ | 1.5 | | 18.0 | ++ ++ ++ | 0.1 | − − − | 0.03 | − − − |
| III (0.05) | 15 | 3 | 7.0 | − − − | 0.8 | | 7.3 | ++ ++ ++ | 0 | − − − | 0 | − − − |
| | 25 | 3 | 13.6 | ++ ++ + | 1.5 | | 8.5 | ++ ++ ++ | 0 | − − − | 0 | − − − |
| | 35 | 3 | 27.5 | ++ ++ + | 3.2 | | 19.0 | ++ ++ ++ | 0.32 | − − − | 0.04 | − − − |

* Number of colonies was expressed per 1 mg. of each organ.

† The same as shown in Table I.

into each of 3 tubes of Ogawa's egg media. After cultivation at 37°C for 4 weeks, the number of colonies was counted.

All the results are shown in Table II.

1) In the course of the experiment only 2 mice died within 35 days.[10]

2) The number of colonies shown in Table II are expressed per 1 mg. of the organ tissue. With lung, liver and spleen materials the colonies were proved early in 15 days, but with lymph nodes and ascitic fluid even 25 days after injection no colonies have been proved, until it was found in a very small number after 35 days.

3) Comparative study of the number of colonies in each group indicated that, when the more inoculum was injected the more colonies were found in every viscera after 15 days.

4) After 15 days, the increasing number of colonies was found in the order of spleen, lung and liver, although no remarkable differences were observed among them. After 25 days the more colonies were found in the order of lung, spleen and liver, namely the bacilli in lung tissue were especially increased in the later period of disease. After 35 days the order is the same as after 25 days. In this experiment it was proved, that the increasing grade of the bacilli in liver seemed to be somewhat slower than in lung and spleen.

5) After 15 days the pathological changes were compared macroscopically. In lung there were no visible evidences of the disease in any group. All of mice were increasing in the weight of spleen already after 15 days except the first group which was injected with 0.01 mg. of bacilli. Macropathological findings were also increasing with the course of disease except lymph nodes and ascitic fluid where no evidence of disease was found until 35 days.

Exp. III. The development of bacilli in liver tissue, examined by repeated resection of liver-pieces.

Sixteen white mice weighing 14–18 g. were devided into 4 groups consisting from 4 mice each. All mice were inoculated intravenously with 0.05 mg. (moist weight) of virulent human-type tubercle bacilli ($H_{37}Rv$).

As shown in Table III, after one week of infection all animals of the 1st group, after 2 weeks those of the 2nd group, after 3 weeks those of the 3rd group, and after 4 weeks those of the 4th group, were operated up for liver resections. The operation was repeated in such a manner, as after 5 weeks the 2nd operation of those of the 1st group was carried out, and under such a schedule the resections were repeated 3–4 times in every animal of all groups, being continued until 16 weeks after in-

TABLE III

The Development of Living Tubercle Bacilli
Number of Colonies Cultivated from
Liver-pieces

The figures indicate the number of colonies, obtained by the cultivation of diluted liver suspensions shown in table

| Group | Lapse of time between inoculation and liver resection (weeks) | Dilution of the material (100 mg/ml) for cultivation | Number of mouse | | | | |
|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | Average |
| I | 1 | $10^{-1}$ | ||| ||| ||| ||| ||| |
| | 5 | $10^{-3}$ | 5.5 | 15.5 | 34.7 | 10.1 | 16.3 |
| | | $10^{-4}$ | 0 | 0 | 2.0 | 1.0 | 0.8 |
| | 9 | $10^{-2}$ | 1.0 | 3.2 | 5.1 | 1.5 | 2.7 |
| | | $10^{-3}$ | 0 | 0 | 0 | 0 | 0 |
| | 13 | $10^{-2}$ | 3.0 | died after operation | died after operation | 6.0 | 4.5 |
| | | $10^{-3}$ | 0.3 | | | 0.5 | 0.4 |
| | | | 5 | 6 | 7 | 8 | Average |
| II | 2 | $10^{-1}$ | ||| ||| ||| ||| ||| |
| | 6 | $10^{-3}$ | 11.5 | 30.5 | 5.0 | 12.5 | 14.9 |
| | | $10^{-4}$ | 1.0 | 4.5 | 1.0 | 1.5 | 2.0 |
| | 10 | $10^{-2}$ | 4.0 | 43.0 | 34.5 | died after operation | 40.8 |
| | | $10^{-3}$ | 0.5 | 8.0 | 1.5 | | 5.0 |
| | 14 | | sacrificed | sacrificed | died after operation | | |
| | | | 9 | 10 | 11 | 12 | Average |
| III | 3 | $10^{-2}$ | 28.0 | 132.0 | 102.0 | 51.0 | 78.5 |
| | | $10^{-3}$ | 2.8 | 13.2 | 10.2 | 5.1 | 7.8 |
| | 7 | $10^{-2}$ | 14.0 | 23.5 | 15.0 | 17.0 | 17.4 |
| | | $10^{-3}$ | 1.0 | 6.3 | 1.5 | 1.5 | 2.6 |
| | 11 | $10^{-2}$ | 0 | 4.5 | 3.5 | 3.0 | 3.7 |
| | | $10^{-3}$ | 0 | 0.5 | 0.4 | 0 | 0.2 |
| | 15 | $10^{-2}$ | 0 | 3.5 | 4.0 | died after operation | 2.8 |
| | | $10^{-3}$ | 0 | 0.4 | 0.4 | | 0.2 |
| | | | 13 | 14 | 15 | 16 | Average |
| IV | 4 | $10^{-3}$ | 26.5 | 66.0 | 49.5 | 46.5 | 47.0 |
| | | $10^{-4}$ | | | 6.5 | 9.0 | 7.8 |
| | 8 | $10^{-2}$ | 31.0 | 154.2 | 83.0 | 23.0 | 72.8 |
| | | $10^{-3}$ | 2.0 | 12.0 | 7.5 | 1.0 | 5.6 |
| | 12 | $10^{-2}$ | 4.0 | 15.0 | 6.0 | 2.0 | 6.8 |
| | | $10^{-3}$ | 0.5 | 1.5 | 0 | 0 | 0.6 |
| | 16 | $10^{-2}$ | died after operation | died after operation | 1.0 | died after operation | 1.0 |
| | | $10^{-3}$ | | | 0 | | 0 |

oculation at last.

From all the pieces of liver, resected in the manner mentioned above, the tubercle bacilli were cultivated immediately after resection as described later.

The weight of liver pieces resected was 50 to 100 mg. each, and occurring hemorrhage was in no need of treatment, except that after the resection peritoneum and skin were sewn up together and 0.5 cc of physi-

ological saline was injected subcutaneously. Accidental deaths by this operation were scarcely observed even after repeated operations as shown in Table III. So it seems to be available for its simplicity and for its quantitative character to pursue the development of bacilli in tissues.

The autopsy of dead animals during the schedule of liver resections was conducted immediately after the death of animals, if any, and that of living animals was carried out at the suitable time, between 1–5 weeks after the last resection, i.e. between 17–21 weeks after inoculation, and the tubercle bacilli from lungs, livers, spleens and both kidneys including the liver pieces resected were cultured according to Ogawa's method.[11] The weighed amounts of materials were ground in 10 volumes of 1 per cent NaOH solution, and diluted further with same solution 10 fold serially as $10^{-1}$, $10^{-2}$, $10^{-3}$, and $10^{-4}$. 0.1 cc. of each diluent was inoculated into 2 or 3 tubes of Ogawa's media.

Results obtained were shown in Table III, IV and V.

1) The moltality of animals by this operations was as follows. After the 1st operation no mice died, and after the 2nd operation died only 1 mouse under 16. But, after the 3rd operation 7 mice died among 15.

2) The maximum number of colonies cultivated from the resected

TABLE IV

Number of Colonies Cultivated from Liver-pieces

| Lapse of time between inoculation and liver resection (weeks) | Number of colonies cultivated from liver-pieces at the dilution of | | | |
|---|---|---|---|---|
| | $10^{-1}$ (100 mg/ml) | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ |
| 1 | ||||| | | | |
| 2 | ||||| | | | |
| 3 | | 76.5 | 7.8 | |
| 4 | | — | 47.0 | 7.8 |
| 5 | | | 16.3 | 0.8 |
| 6 | | | 14.9 | 2.0 |
| 7 | | 17.4 | 2.6 | |
| 8 | | 72.8 | 5.6 | |
| 9 | | | 2.7 | 0 |
| 10 | | 30.5 | 3.3 | |
| 11 | | 3.7 | 0.2 | |
| 12 | | 6.8 | 0.6 | |
| 13 | | 4.5 | 0.4 | |
| 14 | | 3.0 | 0 | |
| 15 | | 2.8 | 0 | |
| 16 | | 1.0 | 0 | |

TABLE V

The Distribution of Tubercule Bacilli and Pathological Findings in Several Organs 6–21 Weeks after Inoculation of Tubercle Bacilli

| Organ | Weeks after the inoculation | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 6(1)‡ | | 10(1) | | 12(2)‡ | | 13(1) | | 14(2) | | 17(1) | | 18(1) | | 19(2) | | 21(1) | |
| | Number of colonies* | Pathological changes† | Number of colonies | Pathological changes | Number of colonies | Pathological changes | Number of colonies | Pathological changes | Number of colonies | Pathological changes | Number of colonies | Pathological changes | Number of colonies | Pathological changes | Number of colonies | Pathological changes | Number of colonies | Pathological changes |
| Lung | 715.0 | ### | 124.5 | ### | 52.4 | ### | 52.0 | ### | 139.5 | ## | 26.0 | ## | 310.0 | ### | 4.3 | ### | 3.0 | ### |
| Liver | 16.5 | | 1.5 | | 1.3 | | 0.3 | | 1.8 | | 0 | | 0 | | 0 | | 0 | |
| Spleen | 106.5 | ### | 10.5 | ## | 8.3 | ### | | ### | 19.8 | ## | 3.0 | ## | 0 | ## | 2.0 | ## | 0.6 | ## |
| Kidney r. | 2.5 | | 1.5 | | 22.5 | | 2.5 | | | | | | | | | | | |
| Kidney l. | 2.5 | | 0.5 | | 0.8 | | 2.0 | | | | | | | | | | | |

* Indicate the bacillary number per 0.1 mg. (wet weight) of each of organs.

† The same as shown in Table I.

‡ The figures in the parenthesis indicate the number of mice examined, and if two animals were autopsied, a averaged number of colonies was listed.

liver-pieces was reached after 4–6 weeks after inoculation, and hereafter decreasing tendency was observed (Table IV). This spontaneous recovery tendency must be fully attended at the judgement of the curative effect of antibiotics.

3) As shown in Table III in details, it seems to be so advantageous that the deviation in the colony numbers in the same group is not so large.

4) The distribution of tubercle bacilli in every organ of the dead or killed animals was presented in Table V. They were autopsied all over 6–21 weeks after the infection and the maximum of the colony number of all materials was proved in case after 6 weeks, and hereafter a decreasing tendency of colonies was proved in these cases too. In any cases the more colonies were found in the order of lung, spleen, liver and kidneys. In kidneys usually some more colonies were found in the right side than the left.

5) Visible pathological changes were most evident in lungs and sometimes many tubercles 1–2 mm. in diameters, were adhered with each other. In liver 2 or 3 smallest tubercles were found usually. These tissues were preserved for histopathological studies.

## Summary

1. 3 experiments in mice tuberculosis, the one with intraperitoneal injection of avian tubercle bacilli (Flemingo strain), and the other two with intravenous injection of human tubercle bacilli ($H_{37}Rv$) were conducted.

In several stages after infection they were sacrificed and the macropathological findings were observed. The development of the disease, however, were chiefly judged by the cultivation test of the bacilli from lung, liver, spleen, lymph nodes and kidneys.

2. For the quantitative pursuance of the living bacilli, the resections of liver-pieces from the tuberculous mice were carried out repeatedly. The accidental death after this operation was not so often, the availability of this method being ascertained.

3. When Flemingo strain was injected intraperitoneally, the bacilli developed chiefly in livers, spleens and lymph nodes, but only little in lungs. Contrary to this intravenous injection of human tubercle bacilli caused the chief lesions in lungs and also in spleens. The natural healing tendency observed in the latter must be attended at the judgment of chemotherapeutic experiment.

In conclution, I wish to express my sincere appreciation to Prof. M. Kuroya for his continued encouragement.

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# Conditioned Galvanic Skin Reflex and Electroencephalogram

By

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(Received for publication, August 18, 1952)

In our previous study on delayed conditioned reflexes,[1] it was found that there appeared two conditioned responses in succession during the application of the conditioned stimulus; the preceding response appeared soon after the onset of the conditioned stimulus and the succeeding one took place with a latency almost equal to the period of delay, i.e. the time interval between the onset of the conditioned stimulus and the administration of the unconditioned stimulus. They were called "initial and delayed responses" respectively.

Any qualitative differences could hardly be found between the initial and the delayed response so far as their behavior in experiment of generalization, differentiation and extinction is concerned. It was demonstrated, however, that these two sorts of conditioned response were different from each other in the central nervous processes as revealed by the electroencephalography. At a certain stage of conditioning the initial and the delayed response were closely related to respective patterns of cortical excitation, i.e. initial and delayed appearance of excitatory waves such as $\beta$ and $\theta$. The delayed cortical excitation became more and more conspicuous as the conditioning advanced while the initial cortical excitation, though most marked at the very beginning of conditioning, became less and less conspicuous to be completely lost sight of at a final stage of conditioning. Based upon this difference we considered that the delayed excitation of the cortex was a true conditioned response of the cortex whereas the initial excitation was not a conditioned response but an unconditioned response to the conditioned stimulus.

Since the initial and the delayed response of the peripheral organ were not essentially different from each other in properties as has been mentioned above, it seems difficult to understand that the cortical processes involved are so different in properties. In spite of our previous

interpretation, the initial cortical excitation may be a conditioned response of the cortex in the same sense as is the delayed one. In the present paper it was shown which interpretation is more probable.

## Experimental

### Methods

The experiment consisted in conditioning the galvanic skin reflex (GSR) using an induction shock applied to the dorsal skin of a foot as an unconditioned stimulus. The method of recording GSR and EEG was not essentially different from that adopted by Motokawa and Huzimori.[2)]

A sound from an electric bell was used as a conditioned stimulus, its duration being fixed at about 15 seconds throughout the present experiment. The unconditioned stimulus was given at the 12th second from the start of the conditioned stimulus.

We selected three employees of our laboratory as subjects of the conditioning experiment. They had no knowledge of the conditioned reflex and were not informed of the conditioning procedure prior to the experiment.

Thus, changes in activity pattern of GSR and EEG in the process of conditioning were traced in order to elucidate the nature of the initial cortical excitation in comparison with that of the delayed one.

### Results

A series of typical records obtained from one and the same subject in one experimental session is presented in Figs. 1 and 2. At the very beginning of conditioning, it was found that the sound stimulus to be used as the conditioned stimulus had an unconditioned excitatory effect upon the subject. As will be seen from Fig. 1A, a GSR was elicited with a latency of about 2 seconds by the onset of the sound stimulus and excitatory waves prevailed in EEG during the application of the stimulus. We, therefore, applied several times the same stimulus at a regular time interval so as to weaken its unconditioned excitatory effect. After this procedure record 1B was obtained. The unconditioned excitatory response of the cortex is obviously less conspicuous in this record than in the preceding one, and the elicited GSR is also smaller in amplitude. Finally, the stage could be reached at which no unconditioned GSR whatever appeared any more and excitatory waves scarcely found in EEG in response to the sound stimulus (Fig. 1C).

The three records presented in Fig. 1 show another remarkable feature. In record 1A, EEG before the onset of the sound stimulus consisted of $\alpha$- and $\beta$-waves frequently mixed with spikes of large amplitude.

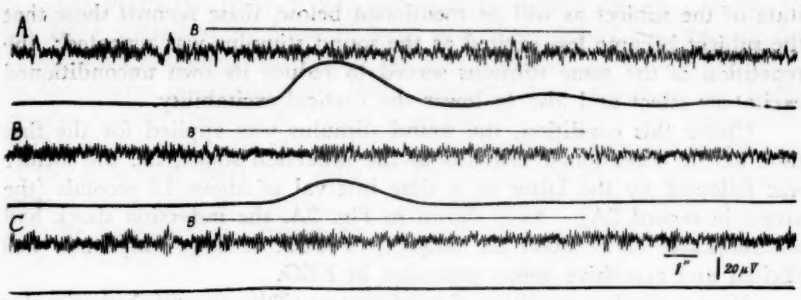


Fig. 1. The sound stimulus was applied repeatedly before the commencement of the conditioning. The line B in all the records indicates the approximate duration of the stimulus. The unconditioned excitatory effect of the sound stimulus upon the electrical activity of the brain and upon the GSR became less marked from record A to C. Note that the spikes prevailing before the application of the sound stimulus in record A could not be found in the corresponding parts of records B and C.

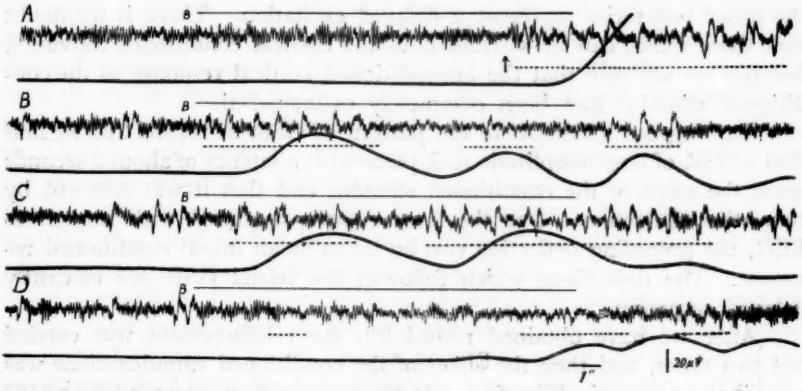


Fig. 2. The process of conditioning. A: The sound stimulus was followed for the first time by the induction shock administered at the arrow. B, C and D: Effect of the conditioned stimulus was examined at successive stages of conditioning. The initial cortical excitation was most marked in record B and decreased in strength until it became very weak in record D, while the delayed excitation attained its maximal strength in record C and still discernible in record D. Note that the initial and the delayed GSR were developed and then weakened almost in parallel with the related cortical excitations.

However, no such spike can be found in the corresponding parts of records 1B and 1C. Since the spikes in question indicate a highly excited

state of the subject as will be mentioned below, these records show that the subject became less excited as the sound stimulus was repeated; the repetition of the same stimulus served to reduce its own unconditioned excitatory effect and also to lower the cortical excitability.

Under this condition, the sound stimulus was applied for the first time as the conditioned stimulus to the induction shock, i.e. the former was followed by the latter at a time interval of about 12 seconds (the arrow in record 2A). As is shown in Fig. 2A, the induction shock had a remarkable effect upon the subject; a GSR of large amplitude was elicited and repetitive spikes prevailed in EEG.

After a single procedure of reinforcement, the conditioned stimulus which was entirely ineffective before the reinforcement became now so effective that there appeared three consecutive trains of spikes in EEG in response to the conditioned stimulus alone (see the parts underlined in Fig. 2B). It is to be noted that the first series of spikes is more marked than the subsequent two series. Taking into account the temporal relation between the conditioned and the unconditioned stimulus, it seems plausible that the first series represents an initial cortical excitation while the other two series represent a delayed excitation. There is no doubt that these spikes can be attributed to the cortical conditioned responses, because we are sure that the unconditioned cortical response to the conditioned stimulus had been completely extinguished.

Turning our attention to the pattern of GSR in record 2B, we find that a GSR of large amplitude took place with a latency of about 2 seconds upon the onset of the conditioned stimulus and that it was followed by two other deflections. For the same reasons as stated with respect to EEG, the preceding deflection can be taken as an initial conditioned response. The deflections which followed the initial GSR are evidently delayed responses.

After we have obtained record 2B, the reinforcement was carried out two times, and then the effect of the conditioned stimulus alone was examined once again (Fig. 2C). It can be seen from records 2B and 2C that the delayed cortical excitation is stronger in record 2C than in record 2B but that the initial one was weaker.

The last record in Fig. 2 (record 2D) was obtained after the reinforcement had been conducted ten times. In this record no initial GSR whatever could be found any more and the cortex ceased to show a marked initial excitation. It is to be noted that the delayed response, central as well as peripheral, develops more slowly and persists longer than the initial response. However, a repeated application of the reinforcement causes a reduction of conditioned responses, as can be seen in record 2D.

Let us survey EEG at the resting level of each record. In record 2A EEG contained no spike of large amplitude before the start of the conditioned stimulus, whereas in the corresponding parts of EEG taken in the course of conditioning there appeared such spikes in the background activity (records B, C and D in Fig. 2). A tentative explanation of this situation may be as follows; on account of the repeated application of the induction shock, the state of the cortical excitation was displaced from a lower level to a higher one and maintained at this level throughout the conditioning experiment.

## Discussion

In the present study of conditioning the GSR, the activity pattern of central as well as peripheral responses has been essentially the same as has been found in the previous study of the conditioned salivary reflexes;[1] the conditioned GSR consists of two separate responses, initial and delayed, appearing in succession during the period of delay, and these two sorts of conditioned GSR are associated with respective cortical excitations. The present study has provided evidence that the initial excitation is no less a conditioned response of the cortex than the delayed excitation. In addition to this, the result has been obtained that the delayed response, central as well as peripheral, develops more slowly than the initial response in the course of conditioning and that the former increased in strength at the cost of the latter. It is also worth noticing that the delayed response, though reduced in strength, can persist after the initial response has been strongly suppressed on account of the repeated application of the conditioning procedure. This finding is essentially the same as has been reported by Motokawa *et al.*[2] who had experimented on the delayed conditioned GSRs.

In regard to the pattern of cortical activity, some remarks must be made. The subject used in the experiment in Figs. 1 and 2 exhibited his cortical excitation in the form of repetitive spikes which could easily be distinguished from the background activity. On account of this fact, it was easy with this subject to trace the changes in activity pattern of the cortex through the course of conditioning. In other subjects employed by us, the cortical excitation manifested itself in the train of $\beta$-waves, no spike of large amplitude being elicited by strong stimulation. In these subjects, the conditioned excitation sometimes can hardly be distinguished from the spontaneous excitation which also appeared in the form of $\beta$-waves. In this case the following criteria can be used so as to make distinction between the conditioned and the spontaneous excitation: The conditioned excitation appears in a certain definite temporal relation to the conditioned stimulus and it takes a protracted

course as compared with the spontaneous excitation.

Rodnick[3] and Switzer,[4] who experimented on delayed and trace conditioned GSRs, obtained the results that the conditioned GSR occurred near the start of the conditioned stimulus at the beginning of conditioning and moved towards the point of reinforcement. These results seem to be contradictory to our findings that two GSRs with different latencies are developed independently and that the initial GSR is gradually extinguished leaving the delayed one near the point of reinforcement. The discrepancy may be explained in the following way. The conclusion of American psychologists has been reached by measuring the mean of the latencies of the conditioned GSRs for twenty or more subjects. From the survey of their protocols it is evident that all the subjects showed the initial GSR at the beginning of conditioning. We may suppose that the extinguishment of the initial GSR accompanying the development of the delayed one occurred in the progress of conditioning at different rates in their subjects. Under this condition, the mean latency of conditioned GSR at a certain stage may be obtained by averaging the latencies of the initial GSRs with those of the delayed ones. Since it is likely that the subject who showed only the delayed GSR increases in number as the conditioning proceeds, the mean latency for all the subjects will increase gradually and reach a certain final value at the end of conditioning.

Supporting evidence of our interpretation will be found in the protocols of Rodnick and Switzer. The protocols show that the increase in the latency of conditioned GSR in some subjects occurred suddenly at one stage of conditioning and amounted to 5 seconds or more, a value too great to be explained as due to experimental errors. We are now convinced that such a sudden increase by a considerable amount in the latency of conditioned GSR is due to the disappearance of the initial GSR and that the increased latencies represent those belonging to the delayed GSRs.

In the present experiment we have noted that the conditioned GSR is formed almost in parallel with the development of the conditioned cortical excitation. On the other hand, it has been pointed out in our previous paper that the conditioned salivary response, especially the initial response, is not yet completely established after the cortex has been well conditioned. From these facts it seems likely that the GSR is more easily conditioned than the salivary reflex. The difference in the rapidity of conditioning may be ascribed to the difference in the susceptibility of the reflex center involved to the cortical excitatory process. Since the salivary center lies far below the cortex in the scale of ontogenetic development than is the center of GSR, it is very likely that the salivary

center is more difficult to be subjected to the cortical excitatory process than is the center of GSR. Thus, it is understandable that the salivary conditioned response is difficult to be detected at the early stage of conditioning whereas the conditioned GSR appears as soon as the cortex is conditioned.

We now discuss the experimental result that the conditioned excitation of the cortex is deteriorated by the repetition of the conditioning procedure. The suppression of the cortical conditioned excitation is supposed to be caused by the inhibitory process acting against the excitatory process. Evidence of this supposition may be furnished by the record in Fig. 2D. In this record, the spikes of large amplitude prevailing in the resting state of the subject disappeared during the application of the conditioned stimulus. From this fact it can be presumed that the cortex which was situated at a higher level of excitation at rest is displaced by the action of the conditioned stimulus to a lower level. As has been advocated by one of us,[5] the downwards displacement of the cortical excitation is an indication of the development of the inhibitory process. On account of this inhibitory process, the initial excitation of the cortex was almost completely abolished and the delayed excitation became less conspicuous. The effect of the inhibitory process was not limited to the cortex, but could reach the center of GSR to suppress conditioned GSRs.

For the same reason as stated above with respect to the difficulty of conditioning the salivary reflex, it may be difficult for the cortical inhibitory process to control the salivary center lying in the lower brain stem. As a matter of fact, we have found that the salivary conditioned reflex, once established, persists longer than the cortical conditioned responses.

From these observations, the conclusion may be reached that, so far as the external reflex response is taken as an index of the cortical process, the GSR can provide more accurate information of the events occurring in the cortex than does the salivary reflex.

The insufficiency of the salivary reflex as an indicator of the cortical process has been considered due to the situation that the reflex center involved is far distant from the cortex in the scale of ontogenetic development. However, the circumstance seems to be slightly different in lower animals. The difference of development between the cortex and the salivary center is evidently smaller in lower animals than in human beings, because the cortex of lower animals is not so highly developed as that of human beings. It is possible, therefore, in lower animals that the salivary center is kept under the control of the cortex in such a way as to reflect the cortical processes in the salivary reflexes faithfully. For

this reason, the salivary reflex has successfully been used as the index of the cortical processes in Pavlov's extensive work on conditioned reflexes in animals.

## Summary

Delayed conditioned reflexes were established in human subjects on the basis of the galvanic skin reflex (GSR) with an electric bell as a conditioned stimulus and with an induction shock as an unconditioned stimulus. The period of delay was fixed at 12 seconds. Changes in the pattern of electroencephalogram (EEG) were traced through the course of conditioning.

1. Prior to the commencement of conditioning, the conditioned stimulus alone was repeatedly applied so as to weaken its unconditioned excitatory effect. After the completion of this procedure, the conditioning was started by applying the conditioned stimulus in association with the unconditioned stimulus.

2. In response to the conditioned stimulus without associating the unconditioned one, there appeared two successive trains of excitatory waves consisting of repetitive spikes or of $\beta$ and $\theta$. The preceding train (the initial cortical excitation) appeared soon after the onset of the conditioned stimulus, while the succeeding train (the delayed cortical excitation) took place near the point of reinforcement. It is obvious in our experiment that these two sorts of cortical excitation represent the conditioned response of the cortex.

3. Just corresponding to the pattern of EEG, the conditioned GSR consisted of two separate responses appearing in succession, i.e. the initial and the delayed GSR.

4. As the conditioning progressed, the initial response, central as well as peripheral, was gradually weakened. The delayed response which was developed more slowly than the initial one in the process of conditioning attained its maximal strength when the initial response began to deteriorate. At the final stage of conditioning, almost no initial response was seen, whereas the delayed response could still persist though greatly reduced in strength.

5. The conditioned GSR was discussed in comparison with the conditioned salivary reflex previously studied by us, and it was concluded that the GSR serves as a more faithful index of the cortical process than does the salivary reflex at least in human beings.

Our sincere thanks are due to Prof. K. Motokawa for his valuable advice during the course of this work.

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# On the ELMoNogram of Cholecystopathia, Preliminary Report

By

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(Received for publication, August 18, 1952)

The following presentation of ELMoNograms[1)3)] is based on the study of 145 cases of cholecystopathia investigated during the past two years in the Gastrointestinal Department of Dr. Mitsuki's Clinic of the Akita Prefectural Hospital. I know that the number of cases of each subgroup was not large enough, so the result would not be fully convincing. But I believe that my own data would be of some use for determing the diagnosis of so called cholecystopathia. Our diagnosis "cholecystopathia" was subdivided into the following 4 groups:—

1. So-called cholecystopathia
2. Ascaris intrusion into the common bile duct
3. Cholelithiasis—only those cases in which stones were found at operation
4. So-called engorged gallbladder (with no stones nor parasites)

The distribution of different Elmonograms according to the 4 sub-

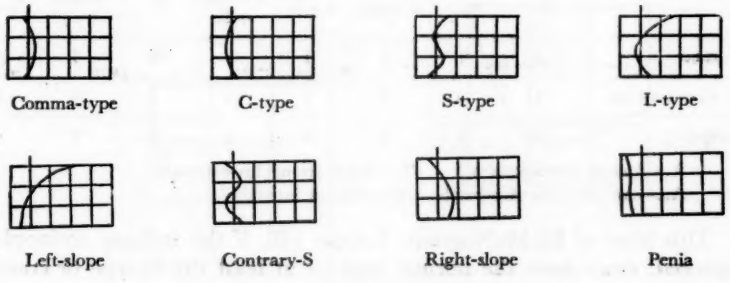



Fig. 1 Types of the ELMoNogram

* Read at the 14th meeting of the Tohoku Session of the Japanese Gastro-enterological Society, Sendai June 1951.

TABLE I

| Types / Diagnosis | Normal | Comma-type | C-type | S-type | L-type L-slope | Contrary S | R-slope | Penia | Total |
|---|---|---|---|---|---|---|---|---|---|
| So-called Cholecystopathia | 10 | 6 | 13 | 6 | 29 | 6 | 4 | 5 | 79 |
| Ascaris invasion | 0 | 1 | 4 | 3 | 8 | 0 | 1 | 0 | 17 |
| Cholelithiasis | 2 | 5 | 9 | 3 | 3 | 2 | 5 | 0 | 29 |
| So-called engorged gallbladder | 5 | 2 | 2 | 3 | 5 | 2 | 1 | 0 | 20 |

groups of cholecystopathia is shown in Table I. Various types of Elmonograms are given in Fig. 1.

It is almost impossible to find a significant difference from the above table, partly because of a rather too small number of cases. Of course it is not to be expected that each of the four subgroups should have a definite Elmonogram of its own, if one takes into consideration various symptoms that each individual case may manifest. Yet I think I can see a certain inclination of the elmonographic curve according to the different subgroups.

1) The Relation between the L-typed Elmonogram of Cholecystopathia and Parasites

In these cases diagnosed clinically as of cholecystopathia and as of L-type Elmonogram, parasites such as ascaris lumbricoides, ankylostoma duodenale, trichostrongylus orientalis and acarus intestinalis were found (Cf. Table II).

TABLE II

| | A. | An. | A.+An. | A.+T. | A.+An. +T. | T. | Ovum (−) | Total |
|---|---|---|---|---|---|---|---|---|
| Normal | 5 | 4 | 2 | 1 | 1 | 0 | 1 | 14 |
| C. Comma-type | 3 | 0 | 3 | 1 | 0 | 1 | 0 | 8 |
| S-type | 0 | 2 | 1 | 0 | 0 | 0 | 1 | 4 |

A.=Ascaris lumbricoides T.=Trichostrongylus orientalis
An.=Ankylostoma duodenale Ac.=Acarus intestinalis

This kind of ELMoNogram- L-type will, if the striking eosinophilia is ignored, draw near the normal type or at least the C-type or comma-type of Elmonogram, neither of the latter two deviating very much from the normal type. Though the cases were small in number, the result was censored by means of the statistical method[4] for case of small number of materials.

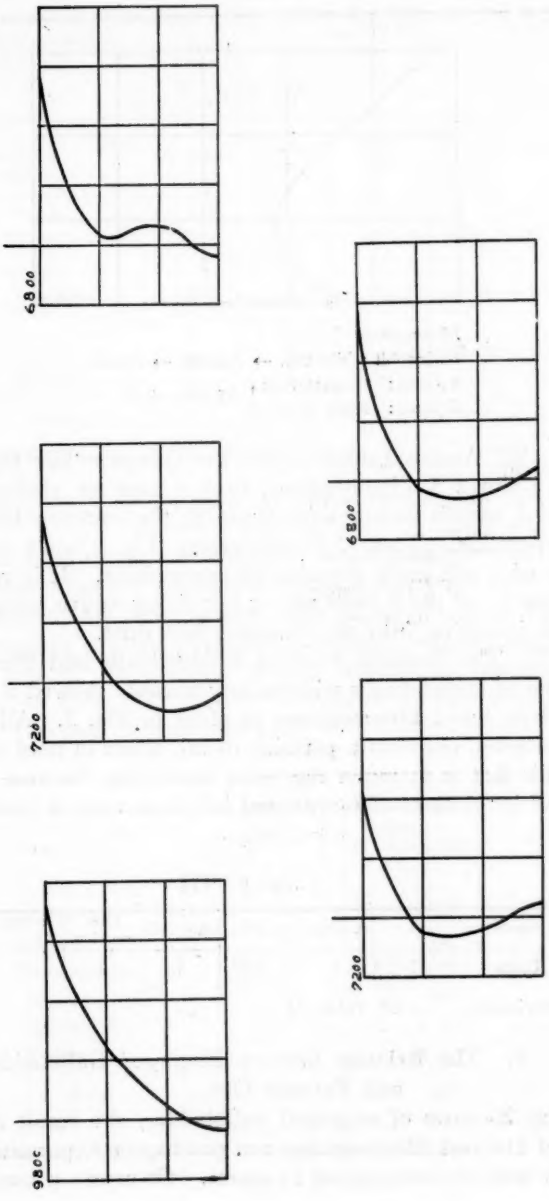
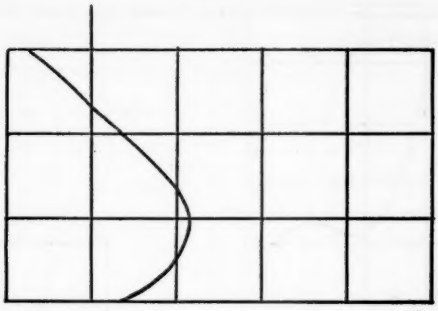



Fig. 2

Fig. 3



Monocytosis
Eosinophil white cell, → Normal → Penia
5 Cases: Comma-type } Modification
3 Cases: Right-slope }

2) Ascaris Intrusion into the Common Bile Duct

Fig. 2 shows 5 Elmonograms, each a case of cholecystopathia in which living ascaris worms were found in the common bile duct at the time of operation. These 5 Elmonograms (Fig. 2) may be regarded as of L-type with still more pronounced eosinophilia. It is very interesting to note that in all the 5 cases one or two living ascaris worms were found in a state intruding into the common bile duct.

3) The Relation between Cholelithiasis and Parasites

8 cases of cholelithiasis without any parasite showed a comma-typed or right-slope typed Elmonograms as given in Fig. 3. All these 8 cases had no parasites, not even a parasitic ovum, either in stool or in duodenal juice. This fact is so much the more interesting, because parasitic ova were found in 15 cases of L-type and left slope type of Elmonogram (Cf. Table III).

TABLE III

| Parasi-teova | A. | A.+An. | A.+T. | A.+AC | Ob-scurity | ovum (−) | Total |
|---|---|---|---|---|---|---|---|
| Cases | 7 | 1 | 2 | 1 | 2 | 2 | 15 |

Abbreviations . . . . Cf. Table. II

4) The Relation between Engorged Gallbladder and Parasite Ova

Among 20 cases of engorged gallbladder, the result shows that, if C-, S- and L-typed Elmonograms are put together, parasitic cases come under this item (8 cases out of 11 cases). Of course a conclusion could

not be drawn due to the small number of cases. Still it is interesting that no case with parasitic infestation came under the item of the normal and right slope Elmonogram.

TABLE IV

| Parasite ova \ Type | Normal | Comma-type | C.S.L.-type | Contrar Y. S. R-slope |
|---|---|---|---|---|
| A. | 0 | 1 | 4 | 1 |
| T. | 0 | 0 | 3 | 0 |
| An.+A. | 0 | 1 | 1 | 0 |
| — | 2 | 0 | 2 | 1 |
| Obscurity | 2 | 0 | 1 | 1 |
| Total | 4 | 2 | 11 | 3 |

Abbreviations . . . . Cf. Table. II

### 5) The Relation between Cholelithiasis and Engorged Gallbladder

The differential diagnosis between cholelithiasis—cholecystopathia with stones—and engorged gallbladder—cholecystopathia with no stones—is very important for practitioners. But as will be seen from Table V, the Elmonogram will not show any difference between the two categories of the disease. It is rather striking that about half the number of cases—55% or 45% (Cf. Table V)—showed an Elmonogram somewhat normal, C-typed or comma-typed.

TABLE V

| Types \ Diagnosis | Cholelithiasis | Engorged-gallbladder |
|---|---|---|
| L-type L-slope | 3 | 5 |
| S-type | 3 | 3 |
| C-type | 16 (55%) { 9 | 9 (45%) { 2 |
| Comma-type | 5 | 2 |
| Normal | 2 | 5 |
| Contrary-S | 2 | 2 |
| R-slope | 5 | 1 |
| Total | 29 | 20 |

Here I may add that those cases of cholelithiasis with a comma-typed or right slope typed Elmonogram prognostically worse than those

with one of the other types. However, I shall report concerning such an inclination with the use of a much larger number of cases in the near future.

## Conclusions

In the present paper the following results will be preliminarily reported.

1. In case of the clinical diagnosis: "Cholecystopathia," "Cholelithiasis" or "Engorged Gallbladder," parasite ova will be found in most cases if the Elmonogram is of L-type and of left slope type. In this respect 5 cases of ascaris intrusion into the common bile duct showed a very definite Elmonogram (Fig. 2).

2. In cases of cholelithiasis and engorged gallbladder with no identified parasite, right slope type of Elmonogram is, it seems, a frequent one.

3. As to cholelithiasis and engorged gallbladder there is, it seems, no type of Elmonogram characteristic of either subgroup.

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# Experimental Studies on Treatment for Liver Disturbances

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Terada[1] and Takezawa[2] of our Clinic reported that fresh tomato-juice (T-juice) was very effective in accelerating the glycogen formation in normal rabbits, and Hisago[3] also confirmed a marked increase of glycogen formation and fixation by the injection of the juice under the condition of high temperature.

In this paper I want to present the studies on the influence of T-juice, sodium thiosulfate (ST) and glucuronic acid (GA) upon the experimental disturbance of the liver.

## Experimental

### Method

Animals employed were adult male rabbits weighing about 2.0-3.0 kg. These animals were fed with 400 g. of *tofukara* (beancurd refuse) and 100 g. of vegetables throughout the period of experiment.

Method of liver function test. Sodium santonate test (S test) and azorubin S test (A-S test) were tried on liver function. The former test was carried out after Takasugi-Miyamoto's method and 0.2 cc. of 5% santonate solution was injected. In the latter test, 2 cc. of 1% A-S solution was injected intravenously and the amount of the dye excreted in the urine in 3 hours was determined by means of Dubosq's colorimeter and the ratio of the amount excreted to that injected was obtained in percentage.

Normal reading was obtained in both tests from the average value of more than three successive experiments when it was constant.

Method of disturbance. Chloroform and tetrachlor carbon (TC) were employed in order to disturb the liver, that is, 0.2 cc. per kg. of body weight was administered subcutaneously in the former and 0.3 cc. per kg. in the latter.

Method of treatment. T-juice for injection was obtained by the following method: fresh and ripe tomatoes were compressed by the compressor and the juice was filtered by means of Chamberland's filter. 1.0 cc. per kg. of body weight was given once a day from the day the liver was disturbed till the recovery from the disturbance.

ST (10% Detoxol) was given by the 3 following methods: a) 0.1 g. per kg. once a day from the day disturbed till the recovery, b) 0.1 g. per kg. once a day for 3 days before the disturbance, c) 0.1 g. in combination with 5 cc. per kg. of 20% glucose solution once a day in the similar way as in a).

The methods of administration of GA are as follows: one is the injection of 50 mg. once a day, the other is that of the same dosage of GA in combination with 5 cc. per kg. of 20% glucose solution.

## Results

1) Influence of T-juice on the liver function disturbed by chloroform

The results of experiment are shown in Fig. 1 in which 0 point indicates the reading before the disturbance and the readings shown on the curve are obtained from the average value of the cases.

*A-S test* (Fig. 1a)

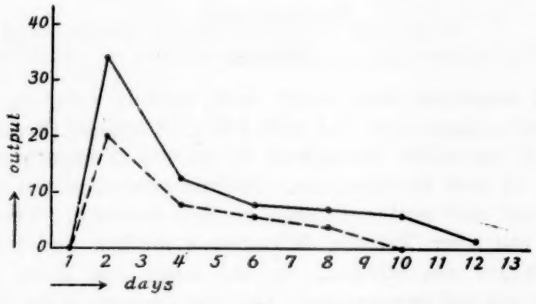


Fig. 1a. Influence of tomato-juice upon liver function disturbed by chloroform, A-S test. —— control cases. - - - - cases given T-juice.

The control experiment was performed in 3 cases disturbed by chloroform. The reading of excretion of A-S returned to the normal in 12, 12 and 14 days and the difference of increase (the difference between the highest reading and the reading before the disturbance) indicated 27, 31 and 44%.

On the other hand, in 3 cases in which T-juice was given after the disturbance, the rading of excretion returned to the normal in 10, 10

and 12 days and the difference of increase was 25, 25 and 29%, showing an improvement of the liver function over the results of control experiment.

*S test* (Fig. 1b)

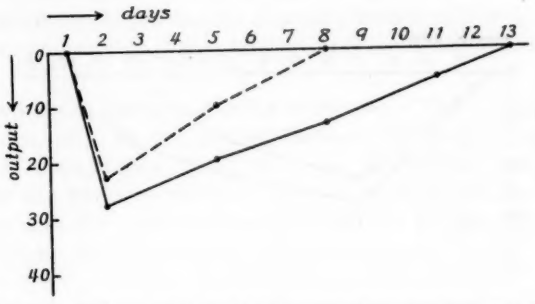


Fig. 1b. Influence of tomato-juice upon liver function disturbed by chloroform, S test. —— control cases. - - - - cases given T-juice.

In two cases in which chloroform was given, the reading of the output of the dye recovered in 11 and 14 days and the difference of decrease (the difference between the lowest reading and the reading before the disturbance) showed 28 each.

In other two cases in which T-juice was given after the disturbance, the output recovered in 8 days each and the difference of decrease was 21 and 25.

2) Influence of T-juice upon the liver function disturbed by TC

The results of experiment are summarized in Fig. 2.

*A-S test* (Fig. 2a)

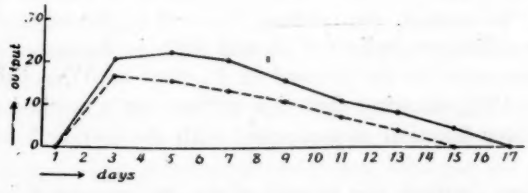


Fig. 2a. Influence of tomato-juice upon liver function disturbed by tetrachlor carbon, A-S test. —— control cases. - - - - cases given T-juice.

From the control experiments done in 2 cases in which TC was given, I found that the reading of excretion of A-S returned to the normal in 16 and 18 days and the difference of increase was 22 and 23%.

On the other hand, in 2 cases in which T-juice was given after the disturbance, the reading of excretion returned to the normal in 15 days each, shortening the time to recover as compared with control cases, and the difference of increase was 16 and 18%.

*S test* (Fig. 2b).

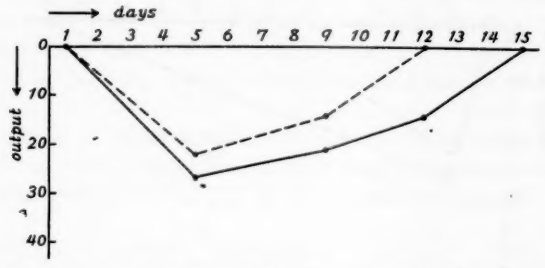


Fig. 2b. Influence of tomato-juice upon liver function disturbed by tetrachlor carbon, S test. —— control cases. ---- cases given T-juice.

In 2 cases in which TC was given, the output of the dye came to normal in 15 days each and the difference of decrease showed 27 and 26.

In 2 cases in which T-juice was given after the disturbance, the period required for recovery to normal was found to be 12 days each and the difference of decrease was 21 and 23.

3. Influence of ST upon the liver function disturbed by TC

*A-S test*

I observed the effect of ST with 3 methods of administration as mentioned above.

In the first method, the amount of A-S in urine returned to the normal in 15 days each and the difference of increase showed 17% each in 2 cases, and in the second, the reading returned to the normal in 13 days each and the difference indicated 14 and 16% in 2 cases. In the third, the reading returned to the normal in 11 days and the difference indicated 15 and 13%, showing that this method has a marked effect in improving the liver function as compared with the former 2 methods.

*S test*

In the first method, the output of the dye recovered in 12 and 15 days and the difference of decrease showed 20 and 19 in 2 cases. In the second, the output recovered in 12 days each and the difference was 24 and 21 in 2 cases. In the third performed in 2 cases, the output returned to the normal in 9 and 12 days and the difference was 17 and 19.

4) Influence of GA upon the liver function disturbed by TC

*A-S test*

In 2 cases in which 50 mg. of GA was given once a day from the day disturbed, the period required for the recovery was 13 days each and the difference of increase indicated 13 and 15%.

On the other hand, in 2 cases in which the same dosage of GA was administered in combination with glucose solution once a day, the reading of excretion recovered in 9 and 11 days and the difference was 12% each.

*S test*

The two same methods of administration of GA as before were employed in this test. In the former method, the output returned to the normal in 12 days each and the difference of decrease was 16 and 18 in 2 cases. In the latter method, the period required for the recovery was 9 days each and the difference was 15 each in 2 cases, showing a good influence on the recovery of the liver function.

## Summary and Discussion

Terada[1] and Takezawa[2] of our Clinic proved that there are some factors besides vitamin C in T-juice which accelerates the glycogen formation, and Hisago[3] also reported that a marked increase of glycogen formation and fixation was observed with the injection of the juice under the condition of high temperature and that the action of the juice was more like that of the mixture of vitamines C, P and K than that of vitamin C only.

I studied the influence of T-juice upon the function of the liver disturbed by chloroform and TC employing both A-S and S tests, and I found that T-juice has a good influence upon the function, that is, the period required for the recovery to the normal was reduced in comparison with the control experiment. Moreover, the results of the experiment showed that the effect of juice was more clearly seen in S test than in A-S test.

These facts suggest us that tomato is effective clinically in improving liver disturbance.

As to the effect of ST on the function of the liver disturbed by TC, the recovery of the function was more rapid in the administration after the disturbance, and its administration mixed with glucose solution after the disturbance was the most effective.

It is generally accepted that GA has an effective action in accelerating the detoxicating function of the liver. From the results of my experiment, the function of the liver was improved with the injection of GA and was recovered more effectively with the administration in combination with glucose solution than GA only.

## Conclusions

1. Tomato juice has a good effect in the recovery of the function of the liver disturbed with chloroform and tetrachlor carbon.

2. Tomato juice exercises a good influence on the detoxicating function of the liver rather than on the excretory function.

3. Sodium thiosulfate is effective in the recovery of the function of the liver disturbed with tetrachlor carbon and is the most effective when administered in combination with glucose solution.

4. The function of the liver disturbed with tetrachlor carbon is improved by the administration of glucuronic acid and its recovery is remarkable with its injection mixed with glucose solution.

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# Studies on the Pathogenesis of Hepato-cerebral Disease.

## I Report. The Determination of Copper in Clinical Materials

By

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Since the appearance of Wilson's original report in 1912,[1] hepatolenticular degeneration has attracted the interest of many clinicial and laboratory workers. This is a rare, familial disease occurring in early life, with the main symptom of extrapyramidal disturbance accompanying mental abnormality and Kayser-Fleischer's corneal ring. It takes a progressive course and ends in fatal outcome within a few years. This clinical process is certainly peculiar, but what makes this disease very interesting is its pathological finding. In the autopsy we may observe simultaneously the degeneration of lenticular nuclei and a sort of portal cirrhosis in the liver. These two organs, which are among the most important parenchymatous organs in the body, have been shown to have close correlation at least in Wilson's disease.

Since the report of Spielmeyer in 1920,[2] Wilson's disease and pseudosclerosis of Westphal and Strümpell are regarded as etiologically identical diseases. Furthermore, Stadler (1936)[3] reported that in the brains of patients suffered from liver disease he observed similar pathological changes as those found in Wilson's disease. Though typical Wilson's disease is relatively rare in Japan, clinicians and pathologists in our country have many opportunities to observe liver disease. Watanabe,[4] Tsubaki, Oyama and Kuroiwa in the Okinaka's Clinic[5] have reported of patients, who after a long course of stenosis of vena cava and liver cirrhosis, developed symptoms similar to those of pseudosclerosis. It has been confirmed in the Okinaka's Clinic that in patients of chronic severe liver involvement, pathological reflexes such as Babinski's phenomenon are more frequently observed than in control persons. Inose[6] reported four

cases of hepatocerebral disease and stated on the ground of clinical and pathological findings that these were to be distinguished from the Wilson-Pseudosclerosis group and to be placed in an intermediate position between this group and atrophic liver cirrhosis. As those reports and numerous other similar literature indicate, many clinicians and pathologists believe that there must exist a hepatocerebral disease or syndrome which would comprehend all the afore-mentioned cases besides typical hepatolenticular degeneration.

Could Wilson's disease be etiologically independent from these hepatocerebral diseases? Are they identical in nature, differing only in their outward appearance? This problem has not yet been settled (even now). Until recently, clinical and pathological observations were relied upon in this research. However, as disturbances of these diseases are believed to be metabolic, biochemical and histochemical researches are now deemed necessary for this purpose besides the former methods of approach. By this means, the etiology of Wilson's disease could also be more directly investigated.

Recently, biochemical researches are reporting of two remarkable findings concerning this disease, namely, metabolic disturbance of copper and amino acid. Both findings seem to be important, but we will now focus our attention only on copper metabolism. Haurowitz (1930)[7] and Cumings (1948)[8] reported that liver and lenticular nuclei of Wilson's disease contained a much larger amount of copper than those of normal control, and Huntington (1951)[9] observed that copper excretion in the urine is increased in patients of the same disease. By using British Anti Lewisite (BAL), copper excretion in urine is increased in Wilson's disease, and Cumings is testing this drug for the therapy of this disease.[10] These findings attracted our attention as they appeared to throw some light on the study of the etiology of Wilson's disease. However, though these authors state that the disturbance of copper metabolism is characteristic in Wilson's disease, it is known that changes of copper metabolism can also take place in some cases of liver disease. For example, Okamoto (1938)[11] reported that copper was detected histochemically in the cells of a certain group of cirrhotic livers. This would seem to indicate the necessity of a more thorough investigation of the copper metabolism in liver and hepatocerebral diseases other than Wilson's cases. Through such an investigation, we should be able to study biochemically the relationships between Wilson's disease, other hepatocerebral diseases and ordinary liver damages, which would lead us to the possibility of a clearer consideration of the etiology of Wilson's disease.

As a preliminary report on the study of this subject, the introduction of a new method of copper determination in clinical materials is essayed in this paper.

## Method

The determination of copper is not practiced in the ordinary clinical laboratory. There exist many varieties of methods of copper determination, which is most perplexing to clinicians, while every method seems to have one defect or another in accuracy, simplicity or availability for clinical research. Fortunately, two Japanese chemists, Dr. K. Kimura and Dr. Y. Murakami have recently reported a new colorimetric method of determination of copper in biological materials[12]. Their method has two points to be recommended: Firstly, that the copper is preliminarily separated from other metals by using a dithizone carbon tetrachloride solution. Secondly, that by the addition of hydroxylamine, the extraction of the colored copper diethyldithiocarbamate complex into the carbon tetrachloride is so rapid and complete that the rerationship between the copper content and the extinction coefficient for the colored matter conforms with Lambert-Beer's Law. Furthermore, the reagents and apparatus required for this method are easily available in ordinary laboratories.

1. *Reagents*

Water: Redistilled in glass apparatus.

70% perchloric acid: Analytical grade.

Concentrated sulfric acid: Analytical grade.

6 *N* hydrochloric acid: Produced from concentrated hydrochloric acid, distilled with an equal amount of distilled water.

0.01 *N* hydrochloric acid.

Concentrated nitric acid: Purified as with the hydrochloric acid.

Saturated ammonia: Obtained by saturating redistilled water with ammonia gas generated from ammonia water by heating with sodium hydroxide.

Carbon tetrachloride: Purified by distillation with dehydrated sodium sulfate.

10% sodium citrate: Prepared as follows; dissolve 20 g. of sod. citrate in 200 cc. of redistilled water, add about 5 cc. of dithizone carbon tetrachloride solution (see below) and shake vigorously. If the color of dithizone changes, remove the dithizone solution and add another 5 cc. of dithizone solution. Shake vigorously as before. Repeat this procedure till the color of dithizone remains unchanged.

0.1% dithizone carbon tetrachloride solution: Dissolve 0.5 g. dithizone in 500 cc. carbon tetrachloride in a separatory funnel of about 1000 cc. capacity. Add about 100 cc. of redistilled water and a small amount of saturated ammonia; shake vigorously. The dithizone, which is green in the carbon tetrachloride, transfers itself into the ammonia alkaline water, changing its color to orange. Remove this dithizone ammonia solution; again add ammonia alkaline water to the residual dithizone carbon tetrachloride solution. Again extract dithizone from carbon tetrachloride into water. Put all the dithizone ammonia water solution into the separatory funnel; and add 500 cc. of distilled carbon tetrachloride and a small amount of 6 *N* hydrochloric acid. Shake vigorously, and as the water changes from alkaline to acid, dithizone changes

its color from orange to green and moves into the carbon tetrachloride. This dithizone carbon tetrachloride solution of about 0.1% concentration is separated from supernatant water and stocked in a colored bottle, shielded from light.

4% hydroxylamine solution: Obtained by dissolving 4 g. of hydroxylamine in 100 cc. of redistilled water.

0.1% sodium diethyldithiocarbamate solution: Obtained by dissolving 0.5 g. of the reagent in 500 cc. of redistilled water and stocked in a colored bottle.

2. *Procedure*

All apparatuses should be free from traces of copper. For this purpose, washing once with diluted nitric acid and twice with redistilled water is sufficient. For testing the presence of copper or other heavy metals, a convenient method is to pour 2 or 3 cc. of dithizone carbontetrachloride solution into the container which is to be examined, and to shake vigorously for about 30 seconds. If heavy metal is present, the color of dithizone changes from green to violet, red or other color.

*Decomposition*

Whole blood or serum: Wet ashing method is used. 3 to 5 cc. of material is measured into a Kjeldahl flask of about 50 cc. capacity, followed by 3 cc. of concentrated nitric acid and 1 cc. of concentrated sulfric acid, and the flask is heated gently on a burner. 3 cc. of perchloric acid is then added, and heating is continued until the remainder of the organic matter is completely destroyed. The content of the tube is removed in a separatory funnel by washing the tube several times with a small amount of redistilled water.

Urine: Urine of one whole day is collected in a copper free bottle. 50 cc. of urine is measured into a 300 cc. Kjeldahl flask, and boiled with 5 cc. of concentrated nitric acid and 2 cc. of sulfric acid. The remainder of organic matter is finally destroyed by the addition of perchloric acid and further heating. The content of the tube is removed into a separatory funnel as described in the case of blood.

Liver and brain tissues: Tissues are dried at 100° C. in an electric thermostatic desicator and weighed. 100 to 200 mg. of dried tissue is placed in a small silica dish and ignited in an electric oven at about 400°C. Heating with a gas flame must be avoided in order to avoid the contamination of copper. The ash is dissolved in 1 cc. of hot 6 *N* hydrochloric acid and run into the separatory funnel. All the contents in the dish are removed completely by washing several times with redistilled water.

*Isolation of copper*

The materials in the silica dish or Kjeldahl flask are removed into a separatory funnel of about 100 cc. capacity. Then add 10 cc. of 10% sodium citrate solution, and some drops of saturated ammonia until the solution becomes a weak alkaline of about 8.5 pH (tested with phenol red paper). Then add the 0.1% dithizone carbon tetrachloride solution and shake for 0.5 minute; draw off into another separatory funnel, leaving a few drops of carbon tetrachloride to prevent transference of the aqueous solution. Continue extraction

in this manner until the last 5 cc. of dithizone solution remains green after being shaken vigorously. All dithizone carbon tetrachloride solutions are collected and are shaken with 10 cc. of 0.01 *N* hydrochloric acid for one minute. If the dithizone remains red after shaking and does not become greenish or purple, keep adding dithizone until the change of color is seen, thus recombining the copper liberated by the treatment. Now draw off the carbon tetrachloride layer into another separatory funnel and shake vigorously for two minutes with 10 cc. of 0.01 *N* hydrochloric acid. Run the carbon tetrachloride into a silica dish; evaporate the solution to dryness on a water bath; add 3 or 4 drops of concentrated nitric acid to moisten the residue; then evaporate and ignite gently to destroy all organic matter.

*Determination of copper*

To the cold dish, prepared as described above, add 1 cc. of 6 *N* hydrochloric acid, and rub over the interior of the dish with a glass rod so that all the cupric oxide will dissolve. Transfer the dissolved material into a separatory funnel, rinsing the dish twice with some redistilled water. Make the solution alkaline of 8 to 10 pH with saturated ammonia. Next, add 2 cc. of 4% hydroxylamine solution and 10 cc. of 0.1% sodium diethyldithiocarbamate solution in the order named. It will instantly become golden yellow. Shake vigorously with 7 cc. of carbontetrachloride and the colored substance will transfer from water into this organic solvent. Draw carbon tetrachloride off into a 25 cc. volumetric flask leaving a few drops; rinse the stem of the funnel with 2 cc. of carbon tetrachloride. Again add 7 cc. of carbon tetrachloride; shake as before and run it into the same flask. Then fill to the mark with carbon tetrachloride and measure the extinction coefficient with a Pulfrich photometer, using a S 43 filter (owing to a derangement in this filter in our instrument, we used a S 47 filter instead). As will be mentioned later, the extinction of the colored matter is so complete that it conforms with Lambert-Beer's Law. Therefore, if a curve of extinction coefficients is prepared for known amounts of copper, we may easily calculate the copper content of the test material from its extinction coefficient.

## Results

*The extinction coefficient of the copper solution*

From a standard solution of 1 mg. of copper per cc., we prepared three kinds of copper solution, containing 5, 10, and 15 gamma of copper per cc. respectively. The extinction coefficients for these solutions and for redistilled water, which were put through the whole procedure described in the above chapter, are shown in Table I. The copper content and its values of extinction coefficient are in linear relationship, thus demonstrating that they conform to Lambert-Beer's Law almost perfectly.

*Recovery ratio of added copper*

As shown in Table II, 3.0 or 5.0 gamma of copper were added to blood or urine. The recovery ratio of these tests was 98 per cent on an average.

TABLE I

Conformability to Lambert-Beer's Law

| Copper present | 0 gamma | 5 gamma | 10 gamma | 15 gamma |
|---|---|---|---|---|
| Extinction coefficient | 0.065<br>0.057<br>0.060<br>0.062<br>0.059 | 0.126<br>0.121<br>0.132<br>0.126<br>0.138<br>0.123<br>0.121<br>0.133 | 0.187<br>0.197<br>0.207<br>0.199<br>0.202<br>0.207<br>0.189<br>0.208<br>0.198<br>0.203 | 0.268<br>0.256<br>0.253<br>0.263 |
| Average of Extinc. coeff. | 0.061 | 0.127 | 0.198 | 0.260 |
| Difference of Extinc. coeff. for 0 gamma and those for 5, 10 and 15 gamma copper | | 0.066 | 0.137 | 0.199 |
| Ratio of the values for 5, 10 and 15 gamma copper | | 1.00 | 2.08 | 3.02 |

TABLE II

Recovery of Added Copper

| Test materials | Amount of copper in material gamma | Amount of copper added gamma | Amount of copper recovered gamma | Recovery ratio (%) |
|---|---|---|---|---|
| Serum of dog (5 cc) | 10.4 | 5.0 | 15.4 | 100 |
| Serum of dog (5 cc) | 10.4 | 3.0 | 13.5 | 103 |
| Whole blood of dog (3 cc) | 6.6 | 5.0 | 11.5 | 98 |
| Urine of man (50 cc) | 7.0 | 5.0 | 11.7 | 94 |
| Urine of man (50 cc) | 19.2 | 5.0 | 24.0 | 96 |
| Urine of man (50 cc) | 4.3 | 3.0 | 7.2 | 97 |
| Urine of man (50 cc) | 8.5 | 5.0 | 13.4 | 98 |

Average value of recovery ratio

*Copper content of blood and serum*

13 cases of human blood and serum were examined. As shown in Table III, there was no marked difference of copper content between

TABLE III

Copper Content of Blood and Serum

| No. | Sex | Age | Disease | Material | Measured copper content | Copper content in 100 cc blood or serum |
|---|---|---|---|---|---|---|
| 1 | male | 31 | healthy | serum | 8.7 gamma/5 cc | 0.174 mg/100 cc serum |
| 2 | female | 28 | Addison's disease | » | 9.2 gamma/ » | 0.184 mg/ » |
| 3 | female | 20 | Grave's disease | » | 8.9 gamma/ » | 0.179 mg/ » |
| 4 | male | 32 | Gastric cancer | » | 12.8 gamma/ » | 0.424 mg/ » |
| 5 | female | 28 | Caries of the spine | » | 11.3 gamma/ » | 0.226 mg/ » |
| 6 | male | 27 | healthy | whole blood | 6.8 gamma/3 cc blood | 0.225 mg/100 cc whole blood |
| 7 | male | 32 | healthy | » | 7.4 gamma/ » | 0.246 mg/ » |
| 8 | female | 20 | Grave's disease | » | 6.7 gamma/ » | 0.223 mg/ » |
| 9 | male | 57 | Peritonitis cartinomatosa | » | 5.3 gamma/ » | 0.177 mg/ » |
| 10 | male | 52 | Banti's syndrome | » | 5.4 gamma/ » | 0.180 mg/ » |
| 11 | the same person as N. 9 | | | » | 11.6 gamma/5 cc blood | 0.232 mg/ » |
| 12 | male | 60 | Gastric cancer | » | 8.3 gamma/ » | 0.166 mg/ » |
| 13 | male | 32 | Gastric cancer | » | 10.5 gamma/ » | 0.212 mg/ » |
| Average value | | | | | | 0.237 mg/100 cc serum |
| | | | | | | 0.208 mg/100 cc whole blood |

blood and serum. The copper content of whole blood was from 0.166 to 0.246 mg. per cent., 0.208 mg. on the average. The copper content of serum is from 0.174 to 0.424 mg. per 100 cc., averaging 0.237 mg.

*Copper content of human urine*

15 cases of healthy persons and of various patients were investigated. As shown in Table IV, there is much more variation of copper content in the urine than in the blood. The amount of copper excreted during a day is from 0.080 to 0.725 mg., 0.266 mg. on the average.

*Copper content of human tissues*

Table V shows copper content in the basal ganglia of the brain, namely, putamen, nucleus caudatus and pallidum, and liver. The tissues were dried in an electric thermostat and weighed. Two materials of tumor tissue in the liver were also studied. No copper was found in them.

## DISCUSSION

In view of the fact that the methods of copper determination itself were still a subject of discussion among biochemists and chemists,[12] we firstly concentrated our attention on the study of the methods and found

TABLE IV

Copper Content in Urine

| No. | Sex | Age | Disease | Amount of urine in one day (cc) | Measured copper content | Copper content in one day's excretion of urine mg. |
|---|---|---|---|---|---|---|
| 1 | male | 32 | healthy | 1,000 | 7.7 gamma/50 cc urine | 0.154 |
| 2 | male | 31 | " | 1,000 | 7.0 gamma/ " | 0.140 |
| 3 | male | 31 | " | 1,000 | 15.6 gamma/ " | 0.312 |
| 4 | male | 25 | " | 1,200 | 9.8 gamma/ " | 0.235 |
| 5 | male | 26 | " | 1,450 | 3.9 gamma/ " | 0.113 |
| 6 | male | 27 | " | 600 | 19.2 gamma/ " | 0.206 |
| 7 | male | 31 | " | 1,150 | 4.3 gamma/ " | 0.098 |
| 8 | female | 29 | Sclerodermis | 350 | 6.8 gamma/10 cc urine | 0.238 |
| 9 | male | 57 | Peritonitis cartinomatosa | 560 | 7.7 gamma/50 cc urine | 0.086 |
| 10 | male | 60 | Gastric cancer | 670 | 16.2 gamma/ " | 0.217 |
| 11 | male | 11 | Narcolepsy | 1,300 | 6.1 gamma/ " | 0.157 |
| 12 | female | 20 | Grave's disease | 2,100 | 8.5 gamma/ " | 0.357 |
| 13 | male | 45 | Liver cancer | 600 | 6.7 gamma/ " | 0.080 |
| 14 | male | 15 | Gigantism | 800 | 11.8 gamma/ " | 0.189 |
| 15 | male | 32 | Gastric cancer | 1,400 | 21.7 gamma/ " | 0.607 |
| The same person | | | | 1,900 | 19.1 gamma/ " | 0.725 |
| Average value | | | | | | 0.266 mg |

TABLE V

Copper Content in Tissues

| No. | Sex | Age | Disease | Copper content in 100 g. dried tissue | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Putamen | Ncl. caudat. | Pallidum | Liver | Hepatom tissue |
| 1 | male | 40 | Hepatom | 5.88 mg.<br>5.18 mg. | 5.28 mg. | 3.70 mg. | | 0 |
| 2 | male | 57 | Peritonitis cartinomatosa | 4.40 mg. | | | | |
| 3 | male | 35 | Uremia | 3.50 mg. | 4.15 mg. | 4.39 mg. | 4.24 mg. | |
| 4 | male | 56 | Hepatom | 4.10 mg. | 3.75 mg. | 3.83 mg. | 2.65 mg. | 0 |
| Average value | | | | 4.61 mg. | 4.39 mg. | 3.97 mg. | 3.45 mg. | 0 |

the one reported by Drs. Kimura and Murakami most satisfactory. We have now turned to the investigation of copper metabolism of Wilson's disease and other liver involvements. Data of these investigations shall be reported later.

We have been studying cholinesterase in connection with biochemical researches on neurology. We have already confirmed the peculiar character of the distribution of cholinesterase in the human brain, the putamen and caudate nucleus having an outstandingly high concentration of cholinesterase compared with that in other parts of the brain.[13] Certainly, cholinesterase is one of the biochemical constituents peculiar to the putamen and caudate nucleus. It is known, and has also been confirmed by us that the level of serum cholinesterase is lowered in patients of liver cirrhosis.[14] However, cholinesterase concentration in the putamen and caudate nucleus seems to show no marked changes in this disease.[15]

If abnormal retention of copper takes place in basal ganglia, it is possible that the metal might inhibit enzyme action, especially that of sulfhydryl groups in these parts. Cholinesterase, one of the enzymes of the sulfhydryl group and contained abundantly in the putamen and nucleus caudatus, might be affected by copper retention. It is our working hypothesis that in Wilson's disease, abnormal retention of copper, caused by or causing liver cirrhosis, also brings about an increase in the copper content of the brain, and that this metal causes most severe damage to the putamen and caudate nucleus through combination with the cholinesterase in these parts, owing to the special affinity of copper to the sulfhydryl group. Several experiments confirming this hypothesis are now in progress in our laboratory.

## Summary

1. The new colorimetric method of determining copper content in biological matter reported by Drs. Kimura and Murakami was studied, and found extremely suitable for clinical investigation of copper.

2. Copper content of whole blood, serum, urine, liver and basal ganglia in normal persons and various patients were investigated.

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# Studies on Brain Stem

## IV. Centering on Fazial Nucleus of Anencephalus

By

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### Introduction

Many instances have been reported on anencephali, all of whom died within a very short time after birth, though lucky enough to survive the parturition, except one case reported by Edinger and Fischer,[1] which lived for a longer period of three and a half years. When an anencephalus lives after birth, be it for a short span, its actions and reactions can be minutely observed during its life-time, and a post-mortem microscopical examination of its central nervous system will furnish a very interesting material for the study of the paths and nuclei of the central nervous system. An embryonic anencephalus is also material of study not far behind an infant anencephalus in scientific interest.

One of the authors of this report, Yamamoto,[2),3)] previously reported on his phylogenetic study In No. II of this series of studies on brain stem, with brains of many kinds of mammalia as materials, and in No. III of the same series, he used brains of human embryoes from 2 to 10 months of age, in an ontogenetic study, to examine the applicability of the famous neurobiotaxis theory by Kappers,[4)] with reference to the facial nucleus in main. In his above studies, he reported, as has been published in this journal, that he found the neurobiotaxis theory hardly befitting the observed facts. We hereunder intend to reexamine the neurobiotaxis theory in its application, using the brain of an anencephalus as material.

According to the neurobiotaxis theory, the nuclei of the cranial nerves, especially, the motor nuclei, are locally transferred due to the neurobiotaxis, during their phylogenetic and ontogenetic development. For example, the facial nucleus, originally situated more cranio-dorsally, is primarily drawn to the caudal from the cranial side by the stimulus exerted "neurobiotaxically" by the solitary tract, and secondarily to the ventral side from a more dorsal position by the strong stimulus from

the developing pyramidal tract, so that the facial nucleus, originally situated more cranio-dorsally, comes to occupy a markedly ventro-caudal position. Kappers and Vogt[5] assert that the neurobiotaxis theory holds good also by anencephali. According to them, when the cerebral hemispheres are utterly absent, the pyramidal tract is also lacking entirely or at most present in only the barest rudiment, so that the facial nucleus is much displaced to the dorsal side from the normal position. However, Toyofuku[6] has found that the position of the facial nucleus was normal, regardless of the degree of the development of the pyramidal tract, as the result of his study on four brains which had their pyramidal tract either in aplastic or agenetic development. Mrs. Bien[7] of the same institute has reported on an interesting study of a malformed guinea-pig. This animal had two brains, the right-side brain of which was nearly perfectly developed, but in the left one, the cerebrum and diencephalon were entirely absent and only a trace of the midbrain was perceptible, the parts from the pons downward being in the relative proportion corresponding to normalcy. The two brains were studied in comparison but no discrepancy from the normal in the position of the facial nuclei was observed in both. Thus, she asserts, the position of the facial nuclei was wholly independent of the development of the pyramidal tract. So, both Toyofuku and Mrs. Bien are not in sympathy with the neurobiotaxis theory.

Divergent views have been propounded on the positions of facial nuclei in anencephali who are utterly unprovided with pyramidal tracts. In the following report, we undertake to set forth our results of observations centering on the position of the facial nucleus, as based on the examination of one anencephalic brain, and simultaneously touch on the problem of the validity of the neurobiotaxis theory.

## Experimental

### Materials

The material used belonged to a ten month human embryo, in which the cerebrum and diencephalon were lacking, and only the parts of the midbrain lower than the inferior colliculus inclusive were found remaining, with only an extremely limited quantity of nerve elements. The relation corresponding to the normal begins to assert itself as far down as the pons. The cerebellum is partially rather well-developed but the other part forms the so-called area cerebro-vasculosa. We studied this material in a series of transverse section preparations, stained with Pal-carmine.

### Findings with Discussion

Most scholars on the subject deny the presence of pyramidal tracts

in brains utterly destitute of cerebral hemispheres. Schwalbe[8] and Zingerle[9] contend that no fibre system can develop, if the seat of the cells of origin, the part concerned of the brain, does not exist, and on this score, deny the existence of pyramidal tracts in anencephali. In the case studied by Edinger and Fischer[1] also the presence of pyramidal tract is negated. On the other hand, Arnold[10] asserts that, though the tract is not visible macroscopically, on microscopical observation, small fibres thereof are always found to exist, so that anencephalic brains even are not utterly destitute of pyramidal tracts.

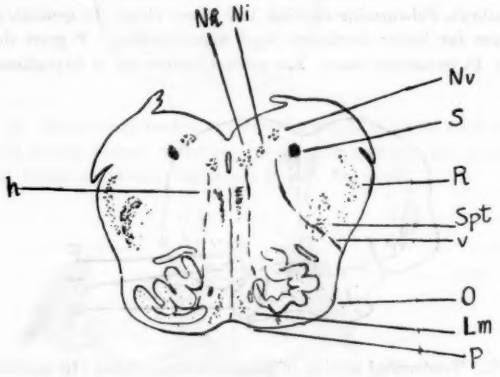


Fig. 1. Transversal section of medulla oblongata of anencephalus (10 month human embryo), Pal-carmine staining, low power view. Pyramid P is entirely undeveloped. O olive; Nh nucl. of hypoglossal nerve; h hypoglossal nerve; Ni nucl. intercalatus; Nv dorsal nucl. of vagus; S solitary tract; v vagus nerve; R restiform body; Spt spinal tract of trigeminal nerve; Lm medial lemniscus.

In our case, as shown in Fig. 1, the olive is considerably well-developed, but the pyramid is entirely undeveloped, the spot appearing concave. In the interior, the medial lemniscus can be clearly traced, though in feeble development, reaching the ventral border of the medulla oblongata, and no trace of pyramidal tract is detected. In collating Fig. 1 with a picture of the corresponding part of a normal 10 month embryo (Fig. 2), it may be seen that the development of fibre system is weak in general, and that the pyramidal tract is entirely lacking. Fig. 3 and Fig. 4 show the incipient and best developed parts of the pons respectively and no trace of pyramidal tract is observable here. In the spinal cord the lateral funiculus where the lateral pyramidal tract should be located is narrower than in normal cases, the nerve elements in the area of the absent pyramidal tract are indistinct and look light throughout, giving a gelatinous ap-

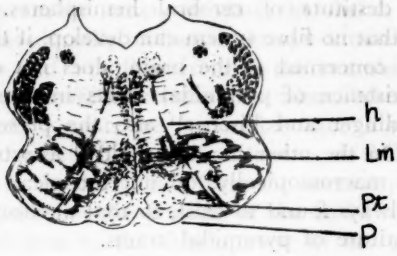


Fig. 2. Transversal section of medulla oblongata of normal 10 month human embryo, Pal-carmine staining, low power view. In general, all nerve elements are far better developed than anencephalus. P good developed pyramid; Pt pyramidal tract; Lm medial lemniscus; h hypoglossal nerve.

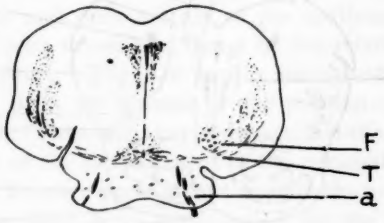


Fig. 3. Transversal section of pons of anencephalus (10 month human embryo), Pal-carmine staining, low power view. No trace of pyramidal tract can be seen. F facial nucleus; T trapezoid body; a abducens nerve.

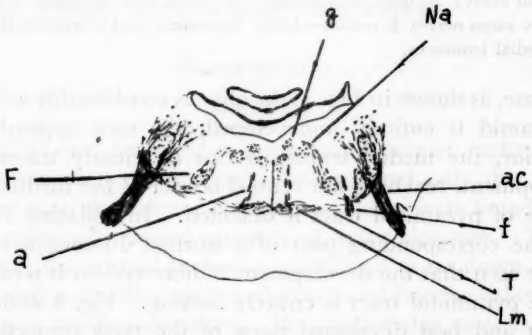


Fig. 4. Transversal section of pons of anencephalus (10 month human embryo), Pal-carmine staining, low power view. No trace of pyramidal tract can be seen and medial lemniscus Lm is weakly developed. F facial nucleus; f facial nerve; g genu of facial nerve; Na abducens nucleus; a abducens nerve; T trapezoid body; ac acoustic nerve.

pearance. Of special interest is the fact that, in the cervical segments, the lateral funiculi are depressed inwardly, toward the place where the pyramidal tract should be located (Fig. 5). That a groove is formed in the direction toward the area where the lateral pyramidal tract should be, in anencephali, has been pointed out by Zingerle[9] in one of his cases

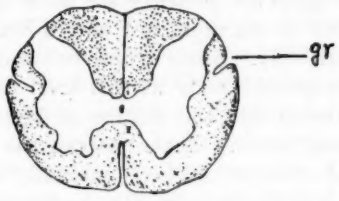


Fig. 5. Transversal section of cervical segment of spinal cord of anencephalus (10 month human embryo), Pal-carmine staining, low power view. At lateral funiculus groove formation gr can be seen.

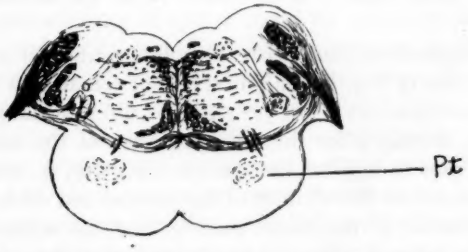


Fig. 6. Transversal section of pons of normal 10 month human embryo, Pal-carmine staining, low power view. Collating with Fig. 4, basilar part of pons is better developed and bundle of pyramidal tract Pt. can be seen.

wherein, he reports, the groove is extended to the inferior parts of the thoracic segments. But in another of his cases, the groove was entirely lacking. Thus, the formation of grooves is not necessarily universal to all spinal cords of anencephali, but this frequent formation is presumably related with the absence or extremely feeble development of the pyramidal tract. The misdevelopment of the pyramidal tract seems to cause not only an abnormal narrowness of the lateral funiculus, but also the formation of grooves due to the physical depression of the surface by the weakened interior resistence.

Now, in our case, in spite of the nearly entire absence of the pyramidal tract, the facial nucleus is located at the very position corresponding to normalcy, as shown in Fig. 3. In normal brains, the nucleus is found

just above in close vicinity of the trapezoid body, and also in our anencephalus, the location is wholly analogous, no swerving toward the dorsal side being perceptible. However, on approaching the cranial extreme from Fig. 3 to Fig. 4, at the latter level the facial nucleus is seen to the side of the dorsal. As at this level the basilar part of the pons becomes stouter—nerve cells are found scattered all over the basilar part, but few, if any, nerve fibres are present— the facial nucleus is at a distance from the ventral border of the pons, but only a little more to the dorsal side from the trapezoid body. Since it may be assumed that the stronger development of the trapezoid body at this level causes a push upward of the nucleus to the dorsal side, this shifting of the position is quite analogous to that of a normal brain. That is to say, the facial nucleus, in approaching the cranial extreme, swerves gradually to the dorsal side at each level, from the location it originally begins its appearance, as the trapezoid body develops stouter. When the superior olive makes the appearance, this nucleus also contributes to the shifting toward the dorsal side. This shifting is entirely of a passive nature, as Toyofuku[6] asserts, and it is quite clear that it is unrelated with the development of the pyramidal tract.

As for the mutual relation with the solitary tract, it may be pointed out that at the level of Fig. 1, the solitary tract remains to be seen at both sides, but the facial nucleus is not yet visible. On gradual progress toward the cranial side, shortly after the disappearance of the solitary tract the facial nucleus begins to appear, first on one side, that is, the facial nucleus begins its appearance in the vicinity of the cranial end of the solitary tract. This local correlation of the facial nucleus and the solitary tract differs nothing from the normal, but as the solitary tract is present as well as in the normal brain, this persistence in the normal position of the facial nucleus has no value as a material in discussing the validity of the neurobiotaxis theory.

An interesting fact found in our case is that the motor nuclei of the cranial nerves and also the nerves originating in those nuclei are well-developed in spite of the absence of the pyramidal tract. In the spinal cord, the ventral roots are well-developed, though the cells in the ventral horns are weaker than in the normal case. This fact seems to indicate that these motor nuclei enjoy an independent development and, consequently, independent functioning to very considerable extent in spite of the absence or rudimentary development of the pyramidal tract, that is, regardless of the influence of the cerebral cortex. Yamamoto[3] has touched on this phenomenon from the stand-point of ontogenetic study in his report No. III of this series. Schwalbe[8] and Zingerle[9] are inclined to the view that, when the part of the brain where the cells of origin

are seated are affected not only the neurons directly connected with these cells of origin are degenerated, but also the secondary neurons connected thereto, though in a weaker degree, but we found nothing to indicate any debilitation of nuclei of the cranial nerves and cranial nerves in our case of anencephalia. At any rate, the considerable development of these motor nuclei is presumably indicative of their good functional capacity. However, since the movements occasioned by these nuclei are not under the influence of the cerebral cortex, it follows that they are not voluntary movements. In fact, according to the records of observations on the movements and behaviors of anencephali who enjoyed a brief existence in life, they sucked the fingers thrusted into their mouths, cried, whimpered, in some cases, drank milk and water, and some were seen making wry faces when pinched forcibly, but most of them were incapable of spontaneous movements, except the case studied by Arnold,[10] which moved the extremities automatically. Zingerle says that a faradization of the area cerebro-vasculosa caused a tonic staring, and assumed, that the impulse was transmitted probably through the motorial rubro-olivary tract, considering the absence of the pyramidal tract. In our case, which is an embryo and nothing of course could be recorded thereof on its behavior in life, and we have to limit our description to the imaginary, it is quite unimaginable that its movement ever came from the impulse of the motor nuclei, such as nucleus ruber and other principal extrapyramidal nuclei, which are non-existent, together with the greater part of the midbrain. If then, the motor nuclei of the cranial nerves function at all, it must take the course of reflexive movements. Looking through the reports of behaviors in life of anencephali, we find that they apparently manifest merely reflexive movements in general, and also that many phases of reflexes are preserved intact in them.

To speak on the sensory elements, in our case, the posterior funiculus in the spinal cord is well-developed and the myelination of its nerve fibres is better developed than in other funiculi. As a part of the cerebellum is present, the tracts connected thereto are also in good preservation. The medial lemniscus, as shown in Figs. 1, 3 and 4, is certainly observable, but only in an extremely weak rudimentary state. But when we consider the total absence of the pyramidal tract in our anencephalus, who is unprovided with the thalamus as well as the cerebral hemispheres, it is of interest that the medial lemniscus is present at all. The sensory nuclei of the cranial nerves and the sensory cranial nerves are considerably well-developed, as are the motor nerves. By the impulse coming from these sensory nerve elements, the motor nerve elements are presumed to cause reflexive movements. However, as the impulses from these sensory nerve elements are not conducted to the cerebral cortex, they cannot possibly

evoke consciousness, and in the observations of all authors, no symptom of bad humor expressing consciousness of pain or hunger has ever been recorded.

Therefore, if neurobiotaxis theory has any validity, in an anencephalus, the motor nerve elements should show some neurobiotaxical function toward the sensory nerve elements, since here reflexive movements prevail, as described above. But if we follow the observations made in our case, the positions and mutual local relation of the motor and sensory nerve elements do not show any difference from the normal case, that is, we see nothing apparent in the phenomena of neurobiotaxis connected with anencephalia. We feel justified in doubting the validity of neurobiotaxis theory on this score, too.

## Conclusion

Upon study of a series of preparations taken from an anencephalic brain, which was destitute of pyramidal tract, we found that the position of the facial nucleus did not differ from that of a normal brain and we came to the conclusion that there is no relation between the position of the facial nucleus and the development of the pyramidal tract. Furthermore, we failed to discover any factual phenomenon that would have indicated the effect of neurobiotaxis between the facial nucleus and other sensory nerve elements. In consideration of such observations, we entertain some doubt against the validity of the neurobiotaxis theory. A transfer in position of a nucleus, if any, would be rather of a passive nature.

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# A Counting Chamber Peroxidase Reaction Method for Erythrocytes. A New Technique for Histochemical Examination of Peroxidase Activity of Erythrocytes

By

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There has hitherto been paucity of literature on the counting chamber method or humid method of peroxidase reaction for blood cells, as compared with the reaction of those cells on film method. Sato and Shoji, in 1928, reported the counting chamber peroxidase method for leucocytes, an application of Sato and Sekiya's peroxidase stain.[2] Sakurada[3] modified this further for the purpose of the differentiation between eosinophiles and pseudoeosinophiles of the rabbit. Minagawa,[1] in 1937, demonstrated the copper peroxidase reaction of frog's red cells by the use of his own modification of Sato-Shoji's method, and besides, he showed that his method could be applied to human erythrocytes in some cases of severe anemia. However Minagawa's method is, so far as my experience goes, not to be applicable to normal erythrocytes; in other words his method is not sensitive enough in human case. So the present paper is the very first within my knowledge to report the counting chamber copper peroxidase reaction method for human erythrocytes, which is applicable not only to anemic blood but also to normal cases.

## Technical Procedure

All reagents are aqueous solutions.

| | | |
|---|---|---|
| Reagent A: | magnesium sulphate ($MgSO_4$ $7H_2O$) | 1 g. |
| | 1% sodium chloride | 10 cc. |
| Reagent B: | 0.05% copper sulphate ($CuSO_4$ $5H_2O$) | 1 cc. |
| | 0.05% acetic acid | 1 cc. |
| | 0.05% glycerin | 1 cc. |
| | 0.01% urea | 1 cc. |
| | 0.01% water soluble eosine | 1 cc. |
| | 2% sodium chloride | 5 cc. |
| Reagent C: | benzidine solution** | 5 cc. |

* Now Professor emeritus

1:100 diluted solution of 3% hydrogen peroxide 1 cc.

Reagent B should be renewed once a week and Reagent C once a day.

**Preparation of benzidine solution:——

Rub 2 g. of benzidine with a few drops of 1% sodium chloride solution in a mortar. Add 100 cc. of 1% sodium chloride solution and filter.

Staining: Draw a solid column of blood up to the mark 0.1 of the erythrocyte pipette (**the content of the bulb must be about 1 cc.**) (Fig. 1, *b*), and after rapid wiping of the end, draw Reagent A up to the mark 1.0 (Fig. 1 *c*), and then draw 0.1 cc. of Reagent B (Fig. 1, *d*). Then wait ten minutes, while rotating the pipette on its long axis between fingers. And then fill up to the mark 101 with Reagent C (Fig. 1 *e*). (And so, the amount of blood required for this procedure is about one milligram and the approximate proportion of the amount of blood and of each reagent is shown by the following equation; Blood : Reagent A : Reagent B: Reagent C=1:10:100:1000).

Then the pipette should be vigorously shaken for three to five minutes just prior to filling the counting chamber.

The peroxidase reaction (or amount of peroxidase blue product) of blood cells reaches its maximum usually within twenty minutes and remains almost constant during the following few days if left standng at room temperature.

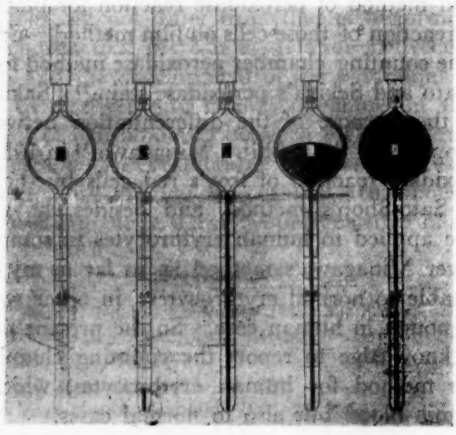

Fig. 1. Staining procedure of the presented counting chamber peroxidase method. The erythrocyte pipette, the content of which is approximately 1 cc., will be filled with blood and reagents successively, as described in this paper. *a*) Pipette without any filling, *b*) Blood filled up to 0.1, *c*) Reagent A (with blood) filled up to 1.0, *d*) 0.1 cc. of Regent B was sucked up and then the pipette was rotated on its long axis between fingers, until ten minutes elapsed, *e*) Filled up to 101.0 with Regent C.

Counting: count one thousand erythrocytes in the counting chamber and find what proportion the following different kinds of red cells show to each other (Figs. 2 and 3).

(1) Peroxidase-negative erythrocytes or aperoxidatic erythrocytes: they look pale and transparent showing nothing distinct but the round

cellular outline (Fig. 3, *a*, 1).

Peroxidase-negative eosinophile erythrocytes or aperoxidatic eosinophile erythrocytes: they appear bright pink and show no peroxidatic granules, so they do not appear blue at all (Fig. 3, *a*, 4).

(2) Peroxidase-positive erythrocytes or normoperoxidatic erythrocytes: they are opaque with light blue color and have fine blue granules in the peripheral part of the cell (Fig. 3, *a*, 2). Such cells represented in Fig. 3, *b*, might be seen as unusual variations of normoperoxidatic erythrocytes.

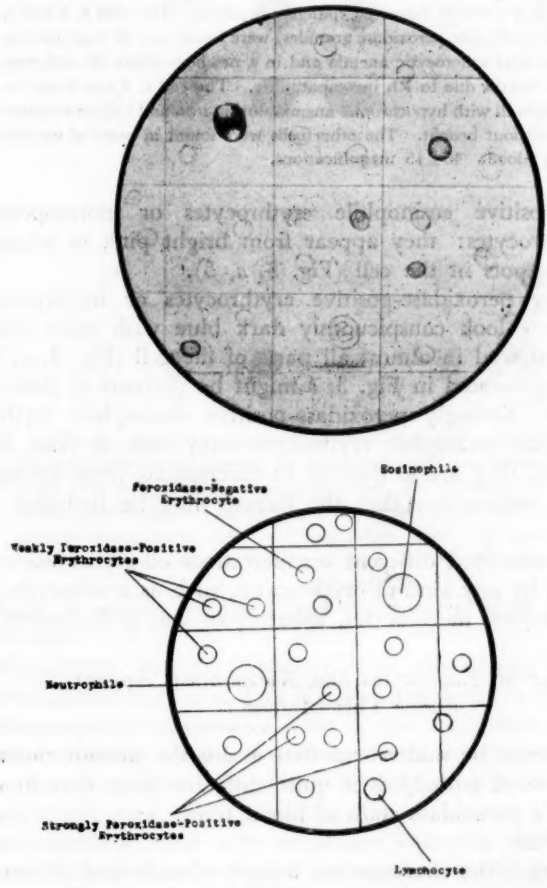


Fig. 2. The peroxidase picture of blood in the counting chamber. 40×10 magnifications.

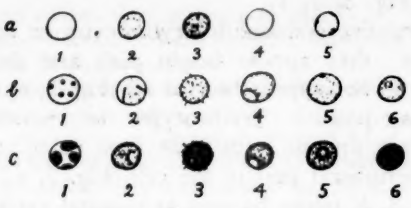


Fig. 3. Peroxidase pictures of erythrocytes. All the cells of Group *a* were found in normal cases. The cells *b* 1 and 2 were found in an 8 year old girl ill with liver cirrhosis following epidemic hepatitis. The cells *b*, 3 and *c*, 3 (somewhat needle-like peroxidase granules) were found in a 10 year old boy ill with congenital spherocytic anemia and in a newborn infant ill with erythroblastosis foetalis due to Rh incompatibility. The cell *c*, 1 was found in a 1 year old girl ill with hypochromic anemia during iron and copper sulphate medication without benefit. The other cells were found in cases of various dyscrasias of blood. 40×15 magnifications.

Peroxidase-positive eosinophile erythrocytes or normoperoxidatic eosinophile erythrocytes: they appear from bright pink to purple with peroxidatic blue spots in the cell (Fig. 3, *a*, 5).

(3) Strongly peroxidase-positive erythrocytes or hyperperoxidatic erythrocytes: they look conspicuously dark blue with more abundant blue granules scattered in almost all parts of the cell (Fig. 3, *a*, 3).

The cells represented in Fig. 3, *c* might be variants of those hyperperoxidatic cells. Strongly peroxidase-positive eosinophile erythrocytes or hyperperoxidatic eosinophile erythrocytes may exist, at least theoretically, but, in fact, they are so difficult to differentiate from strongly peroxidase-positive erythrocytes that the former may be included among the latter.

The above described different manifestations of peroxidase reaction may be revealed by any kind of erythrocytes, such as normocytes, microcytes and macrocytes, megalocytes, spherocytes and poikylocytes.

## How do Leucocytes and Normoblasts Appear under This Method?

At first, it must be understood that under the present method the peroxidase picture of leucocytes is quite different from that in case of Sato and Sekiya's peroxidase stain of blood films.

1. Leucocytes

The following is the characteristic feature of each kind of leucocytes.

Lymphocytes: they are peroxidase-negative and have no blue granules (Fig. 4, *a*). The nucleus appears slightly yellow or colorless and the

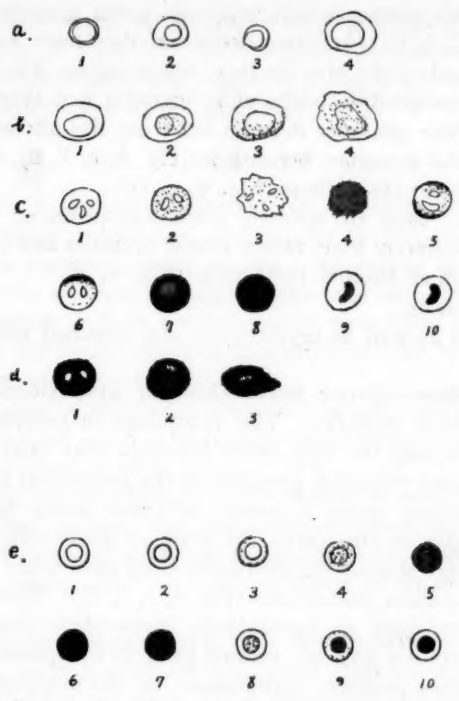


Fig. 4. Peroxidase pictures of leucocytes and normoblasts, showing different enzyme activity. *a*) Lymphocytes, peroxidase-negative. *b*) Monocytes, most cells weakly peroxidase-positive. *c*) Neutrophils, most cells peroxidase-positive in very different intensity or only in nucleus. *d*) Eosinophils, strongly peroxidase-positive. *e*) Normoblasts, most cells peroxidase-positive in very different intensity or only in nucleus. $40 \times 15$ magnifications.

cytoplasm is usually colorless and transparent with occasional yellow granulation.

Monocytes: they find themselves in one of two types, either peroxidase-negative (Fig. 4, *b*, 1) and peroxidase-positive (Fig. 4, *b*, 2, 3, 4). Peroxidase-positive monocytes have a few fine blue granules in their cytoplasm and may have a fewer number of the granules in the nucleus. The outline of nucleus is not clear. Both types of the cell may be stained diffusely slight pink.

Neutrophiles: they also show the following different intensity of the peroxidase reaction. Peroxidase-negative neutrophiles (Fig. 4, *c*, 1) have no blue granules and their pre-existing cytoplasmic granules appear yellow to red. Peroxidase-positive neutrophils have a few blue granules

in their cytoplasm among which yellowish green granules may be seen (Fig. 4, *c*, 2, 3, 4, 5, 6). In other occasions, they have a few granules or are stained diffusely light blue in their nuclei alone (Fig. 4, *c*, 9). The strongly peroxidase-positive neutrophile includes two types, e.g. the one is packed with blue granules densely, covering the all or the almost all part of the cellular structure beneath it (Fig. 4, *c*, 7, 8), while the other is dark blue only in its nucleus (Fig. 4, *c*, 10).

Eosinophiles: they are strongly peroxidase-positive; the cytoplasma is packed with distinctly blue, rather coarse granules and the nucleus may remain unstained or stained light pink (Fig. 4, *d*).

2. Normoblasts

Normoblasts as well as erythrocytes are classified into the following three types.

(1) Peroxidase-negative normoblasts or aperoxidatic normoblasts: they have no blue granules. The cytoplasm is colorless, transparent and shining, reflecting the light more intensely than that of lymphocyte. There may be some yellowish granules in the peripheral part of the cytoplasm. The nucleus seems a round yellowish mass, homogeneous or somewhat granular in structure. In some of those cells, the cytoplasm and the nucleus are sharply differentiated from each other by the presence of a thickened nuclear membrane (Fig. 4, *e*, 1, 2). Peroxidase negative eosinophile normoblasts or aperoxidatic eosinophile normoblasts: they have no blue granules and are stained pink in cytoplasm or nuclei.

(2) Peroxidase-positive normoblasts or normoperoxidatic normoblasts; they have a few fine blue granules either in nuc ei or in cytoplasm. The nucleus is an obvious yellowish mass with or without a few blue granules and the cytoplasm appears rather transparent with scattered a few fine blue granules in it or it seems slightly opaque with faintly blue coloration (Fig. 4, *e*, 3, 4, 5, 6, 8).

Peroxidase-positive eosinophile normoblasts or peroxidatic eosinophile normoblasts: they have a few blue granules either in their nuclei or in cytoplasm or in both and besides they are stained pink.

(3) Strongly peroxidase-positive normoblasts or hyperperoxidatic normoblasts: they are more or less outstandingly blue by the presence of a number of distinctly blue granules either in nuclei or in cytoplasm or in both of them (Fig. 4, *e*, 7, 9, 10). In some of these cells, there may be blue granules so dense and so close to each other in cytoplasm that the outline of nucleus is scarcely recognizable. Strongly peroxidase-positive eosinophile normoblasts or hyperperoxidatic eosinophile normoblasts: they are laden with peroxidase blue granules heavily and besides stained pink in nuclei or cytoplasm.

As far as my own experience goes, normoblasts usually show a rather

symmetrical arrangement of peroxidase blue granules in nuclei or / and in cytoplasm (Fig. 4, *e*, 3, 4, 5, 6) while, on the contrary, leucocytes especially some of neutrophils and monocytes (Fig. 4, *b*, 3 and *c*, 5, 6), show asymmetrical distribution of the granule.

## Comment

Up to date, it is obscure whether the substance which gives above described peroxidase pictures of erythrocytes in this method is the real peroxidase as a chemical entity or not. According to D. Keilin, and E. Hartree[6], the catalase of erythrocyte may also have a peroxidase activity of low order and, in the presence of very small amounts of $H_2O_2$, it may oxidize the lower alcohols to aldehydes, the aldehydes to acids and formic acid to $CO_2+H_2O$.

The blue color of peroxidase-positive granules produced in the blood cells by this method faded and became light yellow to almost colorless within thirty minutes of incubation at 70°C.

The blue coloration of blood cells revealed by this method also faded immediately after adding the concentrated phenylhydrazine aqueous solution or the concentrated hydroxylamine aqueous solution to the stained specimen, and subsequently the inner structure of the cell became visible in detail.

As my hitherto experience shows, the peroxidase reaction of erythrocytes seems to be so delicate and labile, because the present method cannot, if applied on air-dried blood films, make the erythrocytes peroxidase-positive, while (myeloid) leucocytes on them will be stained peroxidase-positive.

## Conclusions

A new technic of the counting chamber copper peroxidase reaction method for human erythrocytes and morphological findings of the peroxidase picture of blood cells, normal and abnormal reds as well as leucocytes, revealed by this method, were described.

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The Tohoku Journal of Experimental Medicine, Vol. 57, No. 4, 1953

# On Sensory Terminations in Oral Cavity Base in Man

By

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(Received for publication, August 28, 1952)

As far as we are aware, there has been no histological study reported hitherto on the innervation of the mucous membrane of the base of the oral cavity, either of man or mammals. One of the authors of this report, Ohgaki,[1] previously conducted a minute study on the innervation of the under surface of human tongue, utilizing microscopic slides prepared by Seto's silver impregnation, and succeeded in obtaining many new informations. In succession to the above, we jointly made a strenuous research on the innervation of the basal surface of the oral cavity, using a series of slides similarly prepared and stained, and arrived at the following results.

As the mucous membrane of the oral cavity base is posteriorly containuous with that of the under surface of the tongue, the histological image of the former resembles strongly that of the latter. Namely, the epithelium is composed of the same low non-cornified stratified flat epithelium in general, and the papillae formation of the connective tissue of lamina propria against the epithelium is generally very poor. Accordingly, the development of nerves, especially sensory nerves must be also weak in this region. That is to say, it would be quite easy to conclude, from the above mentioned histological image, the poor development of the sensory nerves in the mucous membrane of this area.

One of us, Ohgaki, in his previous study succeeded in discovering a rather large number of his so-called taste-bud papillae in the epithelium of the under surface of the tongue. So we undertook a forced scrutiny of the mucous membrane of the oral cavity base, which is similar histologically with the under surface of the tongue, for similar papillae, but we found that, generally speaking, this part does not contain taste-bud papillae, because we encountered such tast-buds only in almost negligible few cases. They are formed in usual one or two in the in particular thinned out epithelium which is caused by the tip of a small papilla juttiug

out very strongly into the epithelium, as shown in Fig. 4. After all, only a very few weakly developed taste-buds are observable here.

Now to turn to the innervation of the mucous membrane of the oral cavity base. The small nerve bundles reaching the lamina submucosa are themselves very few in number, so the plexus formed in this part is also of extremely weak development. The nerve elements consist of fine non-medullated vegetative and thick medullated sensory nerve fibres, the former spreading out in the connective tissue beneath the epithelium to terminate as the Stöhr's vegetative terminalreticulum, standing in tactile innervation for small blood vessels, blood capillaries, connective tissue cells etc.

The sensory nerve fibres rise up through the connective tissue beneath the epithelium, most of them running into the papillae, to terminate in their upper parts. But the number of sensory fibres is so small that such papillae as not provided with sensory innervation are found numerously, that is, only a small minority of papillae contains sensory fibres. Consequently, the mucous membrane of the oral cavity base is much poorer in sensory innervation than the under surface of the tongue.

Furthermore, the terminal formation of the sensory nerve fibres is

Fig. 1

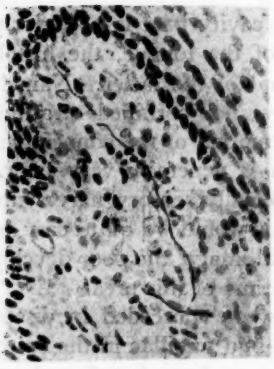

Fig. 2

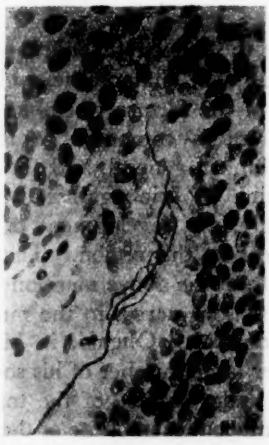

Fig. 1. An unbranched termination found in a small papilla. Mucous membrane of oral cavity base in man. Seto's silver method, × 500, reduced to 2/3.

Fig. 2. An simple branched termination found in a small papilla in mucous membrane of oral cavity base in man. Same staining, × 700, reduced to 2/3.

formed much simpler than that in the under surface of the tongue, the majority being represented as unbranched terminations consisting of minute fibres, running a simple course and terminating sharply right under the epithelium in the tip of the papillae, as shown in Fig. 1. However, there are often seen simple branched terminations of more complex constitution, but they are also composed of fine fibres showing a very simple course and terminating in general sharply in the tip of the papillae (Fig. 2).

As described above, the sensory nerve terminations in the mucous membrane of the oral cavity base are in general of very simple conformation and very small in quantity also, but in very rare cases, some medium sized medullated fibres, upon penetrating the papilla, after losing their myelin and branching out into two or three rami, go over into the glomerular termination, though formed very simply. Fig. 3 shows a typical glomerular termination, from which one nerve fibre is seen running further up to the close vicinity of the epithelium, to terminate sharply. The nerve elements concerning this glomerular formation often show more or less change in size.

In the oral cavity base also were not observed such intraepithelial

Fig. 3

Fig. 4

Fig. 3. A very simple glomerular termination formed in a small papilla. Mucous membrane of oral cavity base in man. Same staining, × 500, reduced to 2/3.

Fig. 4. A glomerular termination formed beneath two taste-buds found in the thin epithelium covering the tip of a small papilla. Mucous membrane of oral cavity base in man. Same staining, × 500, reduced to 2/3.

fibres coming from the sensory terminations described above into the epithelium.

It was imagined that the taste-buds found very rarely in the epithelium of the mucous membrane of the oral cavity base would be of degenerative formations as the majority of those found in the under surface of the tongue, and the development of the sensory nerve fibres for these taste-buds thereto might be very poor. However, it is a remarkable fact that the development of the sensory fibres running towards the taste-buds in this region is often far from poor. As shown in Fig. 4, in the connective tissue directly under two taste-buds a comparatively complex branched termination, especially a quasi-glomerular termination is formed by thick nerve fibres. But, as it seems to be common that also here no existence of intra- and extra-gemmal fibres is observed, these taste-buds are also not of typically sufficient development, and thought to be at least functionally inadequate entities.

## Summary

The development of sensory nerve fibres in the mucous membrane of the oral cavity base is very weak, in parallel with the poor formation of epithelium and papillae in this region, most of the papillae lacking sensory fibres penetrating them. In cases when the papillae are provided with sensory fibres, their terminal formation is generally very simple, being represented by unbranched terminations showing a simple course. But in some cases, the terminations are branched, though simple, and in rarer cases, very simple glomerular terminations are also observed.

In the mucous membrane of the oral cavity base, some few taste-buds can be found, though very rarely. They are formed in the thinned out epithelium, which caused by the papilla sticking into the epithelium. The sensory fibres to the taste-buds are often rather well-developed and sometimes form complex branched termination, especially glomerular termination, but since no fibres are found running into the taste-buds or around them, their functional capacity must be deemed to be of degenerated inadequacy.

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# On Sensory Terminations in Dorsum Manus in Man

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(Received for publication, August 28, 1952)

Many reports on the innervation, especially on the sensory innervation, of the integument have been issued from this laboratory. For example, studies of the hairless skin, especially of the palm by Wada[1] and papilla and areola mammae of human male by Suga,[2] and of the haired skin,—the scalp by Seto,[3] pars cutanea of eyelid by Seto,[4] pars cutanea of lip by Seto, Fujii and Ikui,[5] the abdominal skin by Azuma[6] and scrotum and perineum by Hotta[7]—composed a series of highly interesting achievements, resulting in many a new information.

I undertook the elucidation of the innervation, especially sensory innervation of dorsum manus belonging to the haired skin, in succession of the above illustrious forerunners. The materials were fixed in 10% neutral formol for a long time, cut into 40 $\mu$ frozen vertical sections and stained with Seto's silver impregnation. The series of preparations thus obtained I subjected to minute microscopic examination and arrived at the results described hereunder.

## Individual Views

Since the development of sensory nerve fibres is also in the skin in close relation with the histological feature, I will begin with a histological description of dorsum manus. The cuticle is composed of cornificated stratified flat epithelium continuously falling off, as in the case of common haired skin and the development of the papillae formed by the connective tissue of the papillar layer is very poor, so that even parts without papillae are found. This fact suggests that dorsum manus is very poorly supplied with sensory fibres, as is clear from the many studies reported by this laboratory.

Furthermore, in accordance with the results obtained at this laboratory, the majority of the sensory terminations found in the haired skin

are formed in relation with hairs, so that the parts densely overgrown with hairs are rich in sensory fibres, while on the dorsum manus the inferiority of sensory innervation might be readily surmized, because the downs are very small in number, growing only at the intersections of the sulci cutis.

The hair root of the downs on the dorsum manus is comparatively long and the epithelial and connective tissue sheath around it are rather well-developed, but the development of the sebaceous gland is extremely poor and mostly one-sided in existence. Between the base of the sebaceous gland and the papillar layer is found the arrector muscle.

Now, as the reseaches of this laboratory have established, the development of the sebaceous gland indicates the development of the sensory hair nerve fibres terminating in connection with the hair. Because, as the terminal part of the sensory hair nerve fibres is represented by the special tissue area formed between the sebaceous gland and the outer epithelial sheath, the better the development of the sebaceous gland, the larger is the special tissue area. When the sebaceous gland is only on the one side, the special area is generally formed in a shield shape, representing Seto's hair neuroshield.[3] But when the sebaceous gland is strongly developed so as to surround the outer epithelial sheath in entire circumference, the special tissue area is formed in a shape of atube, corresponding to the Seto's hair neurotube.

The skin nerves to the dorsum manus run through the subcutaneous layer and divide into small bundles in the sudoriferal gland layer lying beneath the reticular layer, to pass into the plexus glandulae sudorifierae. Smaller bundles originated in this plexus penetrate the reticular layer and turn to the connective tissue theca, along the small blood vessels, to end finally in the above mentioned hair neuroshield as hair nerves. However, a small part of these nerve fibres, without entering the hair neuroshield, divert from the main course at the base of the sebaceous gland and running up through the reticular layer of the derm, penetrates into the papillar layer, to end in very simple terminations directly under the epithelium.

The nerve fibres running into the papillar layer are very small in number and the sensory terminations are formed also very simple. That is, the medullated sensory fibres, after losing the myelin, end in unbranched terminations, or in simple branched terminations with only a few branches. Besides, these terminations, as shown in Fig. 1 and Fig. 2, generally do not take a complex winding course, but a comparatively straight course, end mainly sharply and are oftener formed directly under the epithelium outside the papillae, rather than in the papillae proper. On the dorsum manus, as well as in other haired skin areas, it is hardly possible that any

Fig. 1

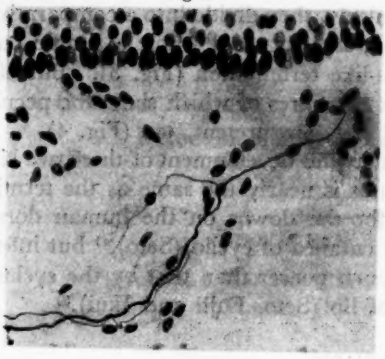

Fig. 2

Fig. 3

Fig. 4

Fig. 1. An unbranched and a bifurcated termination found in the papillar layer beneath the cuticle. Dorsum manus in human adult. Almost no papilla formation seen in this part. Seto's silver method. X 500, reduced to 1/2.

Fig. 2. 2 simple branched terminations in the papillar layer of dorsum manus in human adult. Same staining. X 200, reduced to 3/5.

Fig. 3. Plexus-like termination of sensory hair nerve fibres formed in hair neuroshield (Seto). Dorsum manus in human adult. Same staining. X 320, reduced to 2/3.

Fig. 4. Undetermined termination in hair neuroshield represented by terminal nerve fibres showing marked change in size and winding course. Dorsum manus in human adult. Same staining. X 500, reduced to 2/3.

terminal fibres penetrate into the epithelium to pass into intraepithelial fibres.

The sensory hair nerve fibres always end in the very weakly developed

hair neuroshield described above, that is, reaching the layer of special connective tissue cells arranged circularly, they divide into a comparatively few branches, which run up therein, showing as a whole a simple bush-like arrangement, to form a plexus-like termination (Fig. 3). But often enough, undetermined terminations the fibres of which show the peculiar winding course and the change in size, are present, too (Fig. 4).

The above observations show that the development of these two kinds of terminations on the dorsum manus is nearly the same as the terminal development of hair nerve fibres for the downs on the human dorsum (Sasaki),[8] better than that on pars cutanea of eyelid (Seto),[4] but inferior to that on the scalp (Seto)[3] and much poorer than that by the eyelashes (Seto)[4] and on the pars cutanea of lip (Seto, Fujii and Ikui).[5]

## Summary

In general, in the haired skin, the sensory nerve fibres are mostly represented by sensory hair nerves, the remaining minority ending in the papillar layer. The development of the hair nerves is proportional to the development of the sebaceous gland, and in the papillar layer, the development of sensory fibres is good where the papillae are well-formed. On the dorsum manus, the development of the sebaceous glands are weak and the formation of papillae very poor, so that the sensory nerves cannot be but ill-developed, i.e. the papillae are penetrated by a very few sensory fibres which all end in simplest unbranched and branched terminations. The sensory hair nerve fibres do not go beyond formation of comparatively simple plexus-like or undetermined terminations in the poorly developed hair neuroshields (Seto).

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# On the Hyperglycemia in Man, Produced by Duodenal Introduction of Glucose

By

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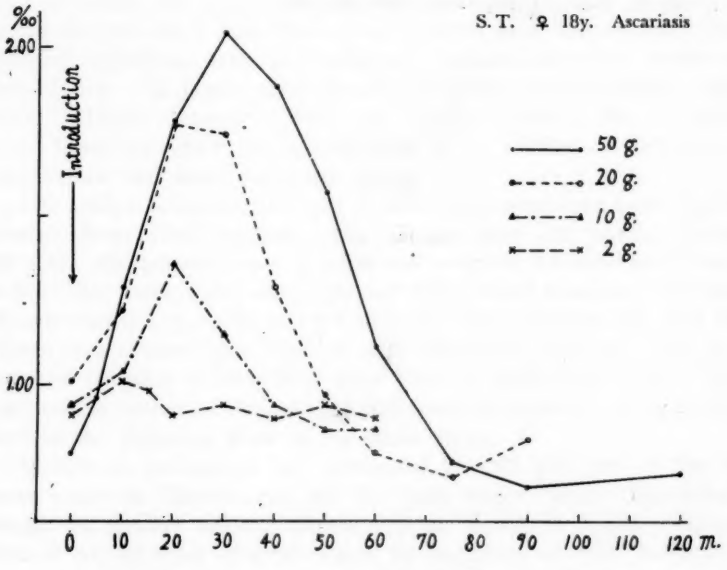
(Received for publication, August 29, 1952)

Variations in the sugar content in the general blood on the introduction of glucose into the duodenum by means of a duodenal tube will be illustrated in the following paragraphs.

The test was done in healthy persons, i.e. those without any disturbances rê the carbohydrate tolerance. In fact this was carried out as the control of the double administration experiments, previously reported.[1)]




Introduction into duodenum of glucose in various doses

The routine diet consists of 440 g. carbohydrate, 90 g. protein and 40 g. fat, and involves about 2500 Calories. In the early morning after the night fast a certain amount of glucose, solved in 100 cc. distilled water and warmed at 37°, was introduced into the duodenum by means of the tube. 2–50 g. were given to 18 individuals.

In general the general blood sugar sets in to incerase two minutes after introduction. 10 g. glucose are enough to see a definite increase in the sugar concentration; the greater the dose, the greater the hyperglycemia and the faster the increase. And when 20 g. glucose is administered the rapidity with which the sugar concentration increases, reaches the maximum, that is the ascending limb of hyperglycemia there wholly coincides with that on 50 g. glucose. The peak of hyperglycemia is however higher in the case of 50 g. glucose than that of 20 g. glucose.

And the duration of hyperglycemia depends also to the doses, and when the dose large enough the hypoglycemia develops finally; the larger the dose, the larger the hypoglycemia.

In short: The blood sugar fluctuations in healthy persons due to the direct administration of glucose into the duodenum are figured according to doses.

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# Studies on Melanin

## XXI. Malignant Melanoma in Japan

By

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In a previous report, several cases of malignant melanoma recently experienced in the author's Clinic were reported and studied chiefly from the histological and histochemical points of view (Itô and Yoshida[1]). In this paper, a statistical study of 206 cases of malignant melanoma collected from Japanese literature, especially of 78 cases originated in the skin was made as compared with that in the white or black races. As the statistical studies of malignant melanoma in Europe we have the treatises by Dieterich[2] (1887) and Luther (1900). Seuqeira and Vint[3] (1934) observed the malignant melanomas in the natives of East and Ventral Africa. In U.S.A. Pack *et al.*[4] (1947) and Macdonald[5] (1948) published statistical data of malignant melanomas from their own points of view. In Japan, there are similar studies by Kawanishi[6] (1928), Miyata[7] (1935), Amano[8] (1941) and Fujita[9] (1948). But no treatise can be found in which the investigation of the differences between the white, yellow and black races was made.

The author collected 206 cases of malignant melanoma from Japanese literature from 1890 through 1949. These cases are mostly Japanese with a few exceptional cases of other yellow races, Chinese and Korean. On the other hand, there were reported 349 cases of malignant melanoma in Connecticut, U.S.A., from 1935 through 1946 (Macdonald), 145 cases in German literature from 1860 to 1882 (Dieterich) and 951 cases at the Memorial Hospital in New York from 1917 to 1946 (Pack *et al.*). From these facts, it cannot be denied that malignant melanomas are more rarely found in the Japanese than in the white races.

Malignant melanoma has accounted for .93 per cent of the total cancer cases in Connecticut for the past twelve years (Macdonald). Though no similar statistic datum can be found in Japan, Nagayo[10] collected 30,782 cases of total cancer by inquiring of chief hospitals in Japan (1933). Therefore it is presumed that the ratio of malignant

melanoma to total cancer in Japan is lower than that in Connecticut.

TABLE I

Incidence of Malignant Melnoma by Site, Sex and Age
(Collected from Japanese literature)

| Site | Total | Male | Female | Under 9 | | 10–19 | | 20–29 | | 30–39 | | 40–49 | | 50–59 | | | 60–69 | | 70+ | | N.S. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | M | F | M | F | M | F | M | F | M | F | M | ? | F | M | F | M | F | |
| Skin | | | | | | | | | | | | | | | | | | | | | |
| Head and neck | 21 | 12 | 9 | | | 1 | 2 | 1 | | 1 | 1 | 1 | | 4 | | 1 | 4 | 4 | | 1 | |
| Upper ext. | 11 | 5 | 6 | | | | | 1 | | | | 1 | 2 | 1 | | 3 | 2 | 1 | | | |
| Trunk | 13 | 11 | 2 | 2 | | | | 1 | | | 1 | 1 | | 4 | | 1 | 3 | | | | |
| Lower ext. | 33 | 22 | 10 | | | 1 | | 1 | 1 | 3 | 1 | 4 | 2 | 7 | 1 | 3 | 4 | 2 | 2 | 1 | |
| Eye | 67 | 30 | 26 | 1 | 2 | 1 | 1 | 1 | 2 | 6 | 5 | 8 | 4 | 11 | | 3 | 1 | 4 | 1 | 5 | 11 |
| Mouth | 24 | 13 | 10 | | | | | 1 | | | 2 | 6 | 3 | 4 | | 2 | 1 | 2 | 1 | 1 | 1 |
| Nose | 18 | 13 | 5 | | | | | | | 1 | 1 | 2 | 1 | 4 | | 2 | 6 | 1 | | | |
| Female genital | 12 | | 12 | | | | 1 | | 1 | | 1 | | 1 | | | 7 | | 1 | | | |
| Others | 7 | 6 | 1 | | | | | 2 | | | | 2 | | | | 1 | 2 | | | | |
| Total | 206 | 112 | 81 | 3 | 2 | 3 | 4 | 8 | 4 | 11 | 12 | 25 | 13 | 35 | 1 | 23 | 23 | 15 | 4 | 8 | 12 |

Table I shows the incidence of malignant melanoma by site, sex and age collected from Japanese literature. There are five cases under nine years of age, three males and two females. Most of them seems to ralated with xeroderma pigmentosum though no detail description about this point could be found in any case.

TABLE II

Incidences of Malignant Melanoma by Sex

| Sex \ Authors | Dieterich | Macdonald | Yoshida |
|---|---|---|---|
| Male (%) | 74 (55.7) | 172 (49.3) | 112 (58) |
| Female (%) | 59 (44.3) | 177 (50.7) | 81 (42) |

Table II is the comparison of the incidences of malignant malanoma by sex as reported by three authors, Dieterich (German, 1887), Macdonald (U.S.A., 1948) and the present author. There is no significant difference by sex in these reports.

The distribution of malignant melanomas by age groups is shown in Table III. There is no significant difference by sex. It occurs most frequently over forty, among males in 77.7 per cent of the cases and among females in 72.9 per cent. Similar data by four authors, Dieterich, Eiselt,

TABLE III

Distribution of Malignant Melanomas by Age Groups

| Age | Males | | Female | |
|---|---|---|---|---|
| | Number | Percentage of distribution | Number | Percentage of distribution |
| Under 9 | 3 | 2.7 | 2 | 2.5 |
| 10—19 | 3 | 2.7 | 4 | 4.9 |
| 20—29 | 8 | 7.1 | 4 | 4.9 |
| 30—39 | 11 | 9.8 | 12 | 14.8 |
| 40—49 | 25 | 22.3 | 13 | 16.1 |
| 50—59 | 35 | 31.3 | 23 | 28.4 |
| 60—69 | 23 | 20.5 | 15 | 18.5 |
| 70 | 4 | 3.6 | 8 | 9.9 |
| Total | 112 | 100.0 | 81 | 100.0 |

TABLE IV

Distributions of Malignant Melanomas by Age Groups (%)

| Age \ Authors | Dieterich | Eiselt | Macdonald | Yoshida | Authors \ Age |
|---|---|---|---|---|---|
| —10 | 4.0 | | .9 | 2.6 | — 9 |
| 11—20 | 1.6 | 5.0 | 3.8 | 3.6 | 10—19 |
| 21—30 | 12.0 | 17.0 | 5.8 | 6.1 | 20—29 |
| 31—40 | 16.0 | 15.0 | 11.9 | 11.9 | 30—29 |
| 41—50 | 23.2 | 25.0 | 14.3 | 19.6 | 40—49 |
| 51—60 | 23.2 | 28.0 | 24.8 | 30.4 | 50—59 |
| 61—70 | 18.4 | 10.0 | 20.4 | 19.6 | 60—69 |
| 70— | 1.6 | | 18.1 | 6.2 | 70— |

Macdonald and the present author, were compared with each other in Table IV. Excepting a fact that the percentage of the cases over seventy years of age reported by Macdonald is significantly higher, no significant difference by each author can be found. The remarkably higher value over seventy by Macdonald seems to be based on the fact that the people over seventy are more in the present U.S.A. than in the past Germany and Japan.

Table V gives the actual numbers and percentages of malignant melanoma in terms of organs primarily affected. It shows that there was a preponderance of involvement of the skin and eye as compared with other regions. Nagayo stated in his clinical statistic studies that skin cancers showed only 177 per cent of 20,782 cases of total cancer. Therefore it is considered that the skin is affected more frequently by

TABLE V

Incidence of Malignant Melanoma in Terms of Organs Primarily Affected (collected from Japanese literature)

| Site | Male (%) | Female (%) |
|---|---|---|
| Skin | 50 (44.6) | 27 (33.3) |
| Head and neck | 12 (10.7) | 9 (11.1) |
| Upper extremity | 5 (4.5) | 6 (7.4) |
| Trunk | 11 (9.8) | 2 (2.4) |
| Lower extremity | 22 (19.6) | 10 (12.4) |
| Eye | 30 (26.8) | 26 (32.1) |
| Mouth | 13 (11.6) | 10 (12.4) |
| Nose | 13 (11.6) | 5 (6.2) |
| Female genital | | 12 (14.8) |
| Others | 6 (5.4) | 1 (1.2) |
| Total | 112 (100.0) | 81 (100.0) |

TABLE VI

Incidences of Malignant Melanoma of the Skin by Site

| Site \ Authors | Dieterich | Luther | Macdonald | Sequeira | Yoshida |
|---|---|---|---|---|---|
| Head and neck | 38 (30.2) | 198 (54.7) | 65 (26.5) | 2 (4.1) | 21 (26.9) |
| Trunk | 40 (31.7) | | 65 (26.5) | 1 (2.0) | 13 (16.7) |
| Upper ext. | 13 (10.3) | 48 (13.3) | 41 (16.7) | 4 (8.2) | 11 (14.1) |
| Lower ext. | 35 (27.3) | 116 (32.0) | 74 (30.3) | 42 (85.7) | 33 (42.3) |

malignant melanoma than by other cancer.

Table VI presents the incidences of malignant melanoma of the skin by site reported by five authors, Dieterich, Luther, Macdonald, Seuqeira and the present author. Sequeira made a study of the natives of East and Central Africa and the present author collected Japanese cases including a few from other yellow races. The other authors showed the data from the white races including a few cases of negroes (three cases in Macdonald's). Lower extremities especially soles have been admitted as the most faboritest location of malignant melanoma. As Table VI reveals, lower extremities are most frequently affected among African ngroes in 85.7 per cent and next often among the Japanese, a yellow race, in 42.3 per cent. Among the white races, on the other hand, the affection of these parts have relatively lower value, 22.8–32.0 per cent. There are differences between three races in the conditions of usual life, e.g. walking with bare feet in the black races. And it cannot be denied that

the influence by the trauma, which seems to occur in different degrees, plays one role in the above-mentioned data. However, if the trauma is the only causative factor in the preponderance of involvement of the lower extremity in malignant melanomas of the skin, a similar fact should be found in the skin carcinoma. As a matter of fact, Tsutusui (Japan, 1910) found only 4.3 per cent of 70 cases of skin carcinoma to occur primarily in either upper or lower extremities. From this, the trauma seems not to be the only and chief cause of the above-mentioned fact. The author thinks it more reasonable to consider this fact as follows: It has been admitted that malignant melanoma are not commonly found in the darker races. (But Sequeira described that it occurs more frequently in the natives of East and Central Africa than in the white races.) It occurs more rarely also in the Japanese than in the white races. In other words, the white races whose skin is scarcely pigmented are more frequently affected by malignant melanoma than the darker races do whose skin is ordinarily deeply pigmented except soles and a few other regions. From this, it can be inferred that malignant melanoma occurs more frequently in the regions which are ordinarily not deeply pigmented and so it occurs relatively equally in all regions of the skin of the white races and very frequently in the soles of the darker races. Thus the author advocates as follows: (1) when the pigmentation occurs in the regions which are not commonly pigmented, it is easy to take malignant cource, (2) pigmented nevus in similar regions has malignant potentiality. The high incidence of melanotic tumors in old gray horses especially in the hoofs or in white cattle is favourable to this advocation.

TABLE VII

Precursor Lesions

| | Number of cases | % |
|---|---|---|
| Naevus pigmentosus (containing lentigo) | 45 | 57.7 |
| Other lesions | 13 | 16.7 |
| no Lesions and not stated | 20 | 25.6 |
| Total | 78 | 100.0 |

Table VII shows precursor lesions of 77 malignant melanomas originated in the skin. In 57.7 per cent of the cases there was a history of previously existing nevus pigmentosus or lentigo and in 16.7 per cent a history was obtained of other skin lesions, e.g. clavus, wart, scar or ulcer. In 25.6 per cent of the cases there was no history of previously

existing lesions or no statement of this point. Dieterich observed a history of nevus pigmentosus or wart in 37, or 25.5 per cent, of 145 cases of malignant melanoma and Luther admitted pre-existent nevus as a precursor lesion in 32 per cent of 471 cases. Driver and Macvicar[11] observed a history of a previously existing pigmented lesion in 48, or 80 per cent, of their 60 cases of Americans containing two negroes. Sequeira, on the other hand, described as follows: " There is no evidence of naevus pigmentosus. In a few instances crab yows was and antecedent, but whether it is causal is not clear." These results, however, are very different depending upon their methods of examination. Therefore, it seems difficult to come to a definite conclusion on the differences between these races.

A history of previous trauma was described in 31, or 39.7 per cent, of 78 malignant melanomas originated in the skin, e.g. medical treatment as excision, incision, electric or medical cauterization and physical irritation as rubbing or scratching. In 47 cases, there was no similar history or no statement of this point. Luther observed a history of trauma only in 32, or 6.9 per cent, of 471 cases, but Driver and Macvicar admitted similar fact in 35, or 58.3 per cent, of 60 cases. However, it must be forgotten that continous irritations may be taken as playing a role unconsciously. The author described above that malignant melanoma occurs more frequently in the regions which are ordinarily not deeply pigmented. But the soles are affected more frequently than the palms (esp. in the black races) though both regions are equally nonpigmented. This can be explained by the fact that the soles receive usualy a continuous irritations especially in the black races. Pack gave a serious consideration only to chronic irritation. The trauma especially continuous irritation seems to take a role, though not single but not chief, in the development of malignant melanoma.

Metastasis was evidenced in 52, or 66.7 per cent, of 78 malignant melanomas originated in the skin. In 19.2 per cent of the cases there was no metastasis and in 14.1 per cent no statement of this point. Macdonald described that among males, 63.9 per cent of the total, not only of the skin, had metastasis and among females, 54.8 per cent had metastasis. Sequeira, on the other hand, described that metastases occur in African negroes but they are far rarer than in Europeans. However, different authors used different examinations and so it seems to be unreasonable to conclude from only this point that this disease is " locally malignant (Sequeira) " in the darker races.

The death of the patient was reported in 24 cases, or 30.8 per cent, of 78 malignant melanomas originated in the skin, and only 3 cases were reported as cured. In 51 cases, there was no statement of this point. Since the methods of observation of the different authors of the prognosis

are very different from each other, it seems to be impossible to compare these data with one another. However, it is supposed that the last group contains not a few cases which must be transferred to the first group. Therefore, it is advocated that malignant melanoma in the Japanese as a yellow race connot be considered to be " locally malignant."

## Summary and Conclusion

A statistical study on 206 cases of malignant melanoma collected from Japanese literaure from 1890 to 1949 was made as compared with those in the white or black races.

1. Malignant melanomas are more rarely found in the Japanese than in the white races and the ratio of this disease to total cancer in Japan is lower than that in Connecticut (U.S.A.).

2. The incidence of malignant melanoma by sex shows no significant difference in both the yellow and white races. The distribution of this disease by age groups presents no significant difference in two sexes and the highest percentage over forty years of age in both the yellow and white races.

3. The incidences of malignant melanoma of the skin by site in the white, yellow and black races were compared with each other. Lower extremities especially the soles which are ordinarily nonpigmented are most frequently affected among African negroes and next often among the Japanese, yellow race. Among the white races, however, the affection of these parts have relatively lower percentage. It is admitted, on the other hand, that the white races whose skin is scarcely pigmented are more frequently affected by malignant melanoma than the darker races do whose skin is ordinarily deeply pigmented except soles and a few other regions. These facts cannot be expalined only by the influence of the trauma, which seems to occur in different degrees in these races. The author advocate as follows: (1) when the pigmentation occurs in the regions which are not commonly pigmented, it is easy to take malignant cource, (2) pigmented nevus in similar regions has malignant potentiality.

4. The problems of precursor lesions, history of previous trauma, metastasis and prognosis were studied statistically. Since the results are very different depending upon the methods of examination, it is difficult to come to a definite conclusion showing the differences between the races. However, it is supposed that malignant melanoma in the Japanese cannot be considered to be " locally malignant."

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# Insulin Sensitivity in Diabetes Mellitus

By

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While the invention of insulin has brought an epoch-making progress in the treatment of diabetes mellitus,[1] insulin resistance in this disease is reported to be on the increase. Accordingly, there is much literature in which classification of diabetes mellitus has been tried from insulin sensitivity.[2]

Falta and his associates have done a great deal in this line; for example, insulin tolerance test[3] and insulin glucose equivalent test[4] were advocated, and especially blood sugar depression curve after insulin injection, described first by Radoslav[5] (1924) and known by his name, has been widely used.

On the contrary, Himsworth[6] and Griffiths[7] used simultaneous glucose-insulin test to assess insulin sensitivity. For superiority of their method they discussed several times with each other.[8]

In this paper the insulin sensitivity in diabetes mellitus were studied by the examination of insulin depression curves, for which a new index was contrived as a criteria.

## Experimental

### Method

47 cases of diabetes mellitus were examined, 20 of which belonged to Type I, remaining 27 to Type II after the classification of our Clinic.[9][10] 3 cases of renal glycosuria and 2 cases of healthy persons were also examined.

The patient received no food nor insulin after supper the previous evening and the test was performed the next morning.

In all the cases 5 units of insulin (" Miniglin ") were injected subcutaneously. The blood sample was taken from the ear lobe before insulin injection and at every 30 minutes for 5 hours after injection. The urine sugar was also examined every one hour.

TABLE I
Total Cases

| Type | No. | Sex | Age | Initial blood sugar value (mg.%) | Depression magnitude (mg. %) | Depression time (hours) | Depression quotient | Blood pressure (mg.Hg.) max. | Blood pressure (mg.Hg.) min. | Body weight (kg.) | Body length (cm.) | Quotient between weight and length |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | ♂ | 42 | 445 | 243 | 4½ | 54.0 | 140 | 80 | 41 | 147 | 27.9 |
| | 2 | ♂ | 42 | 407 | 214 | 5 | 42.8 | — | — | 42 | 147 | 28.6 |
| | 3 | ♀ | 17 | 381 | 305 | 4½ | 67.8 | — | — | 23 | 141 | 16.3 |
| | 4 | ♂ | 14 | 322 | 225 | 3 | 75.0 | 110 | 82 | 28 | 143 | 19.6 |
| | 5 | ♂ | 46 | 312 | 126 | 4 | 31.5 | 146 | 86 | 44 | 163 | 27.0 |
| | 6 | ♂ | 44 | 291 | 183 | 4 | 45.8 | 112 | 70 | 41 | 152 | 27.0 |
| | 7 | ♂ | 50 | 277 | 70 | 3 | 23.3 | 115 | 90 | 63 | 161 | 39.1 |
| | 8 | ♀ | 25 | 273 | 95 | 4½ | 21.1 | 122 | 88 | 53 | 158 | 33.5 |
| | 9 | ♀ | 29 | 271 | 146 | 2½ | 58.4 | 160 | 80 | 39 | 136 | 28.7 |
| | 10 | ♂ | 43 | 271 | 127 | 5 | 25.4 | 140 | 94 | 54 | 161 | 33.5 |
| | 11 | ♀ | 62 | 256 | 157 | 3½ | 44.9 | 120 | 90 | 37 | 148 | 25.0 |
| | 12 | ♀ | 21 | 253 | 143 | 4 | 35.8 | 120 | 90 | 41 | 147 | 27.9 |
| | 13 | ♂ | 38 | 250 | 122 | 3 | 40.7 | 112 | 70 | 48 | 163 | 29.4 |
| Type II | 14 | ♀ | 28 | 245 | 179 | 1½ | 119.3 | 95 | 60 | 30 | 148 | 20.3 |
| | 15 | ♂ | 52 | 233 | 154 | 4½ | 34.2 | 106 | 80 | 51 | 154 | 33.1 |
| | 16 | ♀ | 16 | 232 | 183 | 2½ | 73.2 | 122 | 102 | 25 | 141 | 17.7 |
| | 17 | ♂ | 65 | 228 | 157 | 1½ | 104.7 | 172 | 104 | 57 | 162 | 35.2 |
| | 18 | ♀ | 27 | 225 | 129 | 3½ | 36.9 | 98 | 80 | 35 | 141 | 24.8 |
| | 19 | ♂ | 57 | 219 | 118 | 4½ | 24.0 | — | — | 43 | 157 | 27.4 |
| | 20 | ♂ | 37 | 219 | 51 | 4½ | 11.2 | 136 | 84 | 51 | 167 | 30.5 |
| | 21 | ♂ | 60 | 210 | 154 | 2 | 77.0 | — | — | 53 | 159 | 33.3 |
| | 22 | ♀ | 49 | 206 | 88 | 2½ | 35.2 | — | — | 46 | 150 | 30.7 |
| | 23 | ♀ | 52 | 186 | 94 | 1½ | 62.7 | — | — | 49 | 147 | 33.3 |
| | 24 | ♀ | 69 | 184 | 85 | 1½ | 56.7 | 150 | 92 | 37 | 143 | 25.0 |
| | 25 | ♀ | 61 | 174 | 104 | 3 | 34.7 | 204 | 110 | 45 | 146 | 30.8 |
| | 26 | ♂ | 44 | 158 | 94 | 2 | 47.0 | 106 | 68 | 36 | 162 | 22.2 |
| | 27 | ♂ | 69 | 155 | 51 | 3 | 17.0 | 144 | 80 | 63 | 159 | 39.6 |
| | 28 | ♂ | 57 | 205 | 144 | 3 | 48.0 | 132 | 84 | 53 | 171 | 31.0 |
| | 29 | ♂ | 51 | 197 | 94 | 3½ | 26.9 | 166 | 90 | 58 | 165 | 35.2 |
| | 30 | ♂ | 58 | 181 | 82 | 3 | 27.3 | 160 | — | 57 | 156 | 36.5 |
| | 31 | ♂ | 44 | 172 | 73 | 3 | 24.3 | 110 | 70 | 65 | 158 | 41.1 |
| | 32 | ♂ | 58 | 165 | 57 | 4 | 14.3 | 114 | 70 | 68 | 163 | 41.7 |
| | 33 | ♂ | 75 | 164 | 97 | 1½ | 64.7 | 152 | 70 | 52 | 152 | 34.2 |
| | 34 | ♀ | 64 | 164 | 89 | 3 | 29.7 | 190 | — | 51 | 142 | 35.9 |
| | 35 | ♀ | 50 | 157 | 98 | 2 | 49.0 | 160 | 100 | 56 | 152 | 36.8 |
| | 36 | ♂ | 57 | 148 | 66 | 1½ | 44.0 | 146 | 84 | 63 | 159 | 39.6 |
| Type I | 37 | ♀ | 52 | 148 | 89 | 2½ | 35.6 | 116 | 80 | 59 | 151 | 39.1 |
| | 38 | ♂ | 42 | 146 | 95 | 1½ | 63.3 | 162 | 88 | 46 | 155 | 29.7 |
| | 39 | ♀ | 16 | 134 | 44 | 2 | 22.0 | 98 | 36 | 35 | 148 | 23.6 |
| | 40 | ♂ | 46 | 134 | 56 | 4 | 14.0 | 158 | 82 | 35 | 150 | 23.3 |
| | 41 | ♀ | 51 | 122 | 40 | 1 | 40.0 | 130 | 80 | 54 | 149 | 36.2 |
| | 42 | ♂ | 55 | 122 | 38 | 1½ | 25.3 | 160 | 75 | 55 | 155 | 35.5 |
| | 43 | ♀ | 21 | 115 | 30 | ½ | 60.0 | 154 | 58 | 44 | 145 | 30.3 |
| | 44 | ♂ | 54 | 115 | 14 | 1 | 14.0 | 128 | 87 | 68 | 159 | 42.8 |
| | 45 | ♂ | 63 | 113 | 49 | 2 | 24.5 | 128 | 76 | 46 | 165 | 27.9 |
| | 46 | ♂ | 56 | 102 | 10 | 1½ | 6.7 | 214 | 130 | 69 | 159 | 43.4 |
| | 47 | ♀ | 20 | 84 | 16 | ½ | 32.0 | 124 | 54 | 54 | 151 | 35.8 |
| Renal glycosuria | 48 | ♂ | 38 | 110 | 18 | 1 | 18.0 | 152 | 94 | 60 | 170 | 35.3 |
| | 49 | ♂ | 17 | 96 | 35 | 1½ | 23.3 | 110 | 70 | 37 | 162 | 22.8 |
| | 50 | ♂ | 30 | 94 | 33 | 1 | 33.0 | 146 | 84 | 54 | 165 | 32.7 |
| Healthy persons | 51 | ♂ | 25 | 102 | 17 | ½ | 34.0 | — | — | — | — | — |
| | 52 | ♀ | 26 | 87 | 40 | 1 | 40.0 | — | — | 43 | 153 | 28.1 |

The blood sugar was estimated by Fujita-Iwatake's method,[11] and the urine sugar after Bertrand.[12]

## Results

Without exception insulin caused a depression of the blood sugar. A detail description would be too voluminous to be listed here. Accordingly, the results are summarized in Table I. In Figs. 1 and 2 are shown typical curves from each Type.

The magnitude of the depression curve, which is shown by the difference between the prime blood sugar value before insulin injection and the lowest value after the injection, ranges from 243 to 51 mg.% in Type II, and from 144 to 10 in Type I.

The depression time, at which the maximum drop of the blood sugar occurs, ranges from 1½ to 5 hours in Type II and from ½ to 4 hours in Type I.

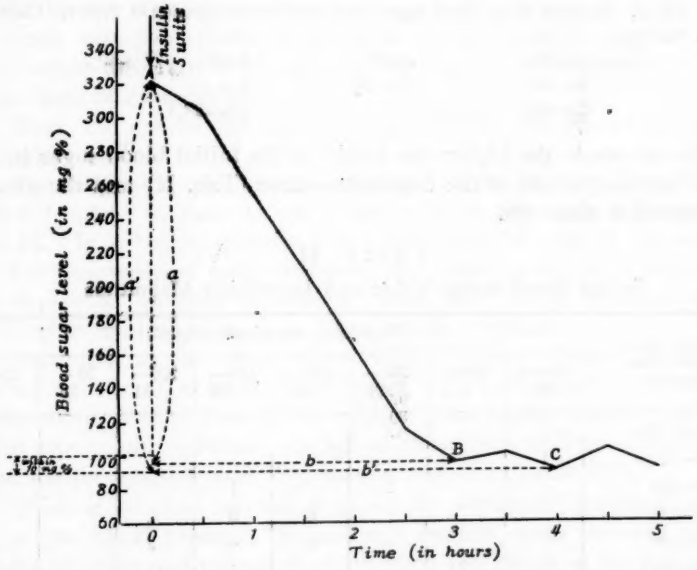


Fig. 1. Example of the blood sugar curve after insulin injection in Type II. Case No. 4.

A=322 . . . . . Initial blood sugar value (mg.%). B=97 . . . . . The lowest blood sugar value after insulin (mg.%). C=92 . . . . . The lowest blood sugar value found on the curve (mg.%). a'=230 . . . . . Magnitude of the blood sugar depression found on the curve (mg.%). b'=4 . . . . . Depression time found on the curve (hours). a=225 . . . . . Magnitude of the blood sugar depression after insulin (mg.%). b=3 . . . . . Depression time after insulin (hours). a/b=75 . . . . . Depression quotient

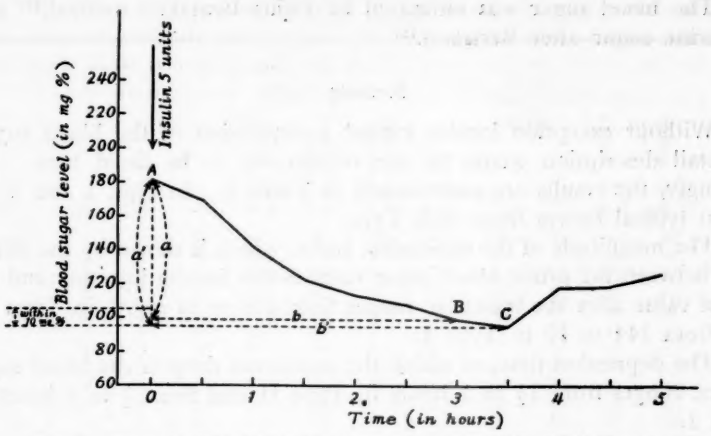


Fig. 2. Example of the blood sugar curve after insulin injection in Type I. Case No. 30.

| | | |
|---|---|---|
| A=181 | a'=85 | a=82 |
| B= 99 | b'= $3\frac{1}{2}$ | b= 3 |
| C= 96 | | a/b=27.3 |

On the whole the higher the height of the initial blood sugar is, the greater the magnitude of the depression curve (Tab. II) and the greater the depression time are.

TABLE II

Initial Blood Sugar Value and Depression Magnitude

| Initial blood sugar value (mg.%) | Depression magnitude (mg.%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Above 301 | 300—251 | 250—201 | 200—151 | 150—101 | 100—51 | 50—26 | 25—0 |
| Above 401 | | | 2 | | | | | |
| 400—351 | 1 | | | | | | | |
| 350—301 | | | 1 | | 1 | | | |
| 300—251 | | | | 2 | 3 | 2 | | |
| 250—201 | | | | 5 | 4 | 2 | | |
| 200—151 | | | | | 1 | 11 | | |
| 150—111 | | | | | | 4 | 5 | 1 |
| Below 110 | | | | | | | 3 | 4 |

It can easily be seen that if the initial blood sugar level is high, then the extent to which the blood sugar can be decreased, will be correspondingly large, and, accordingly, the magnitude of the depression curve and the depression time will be increased. The magnitude of the de-

pression curve does not show the insulin sensitivity.

This consideration leads me to suggset that the insulin sensitivity must be shown by the velocity which can be produced from depression time and depression magnitude. Accordingly, as a criteria for the estimation of insulin sensitivity, I contrived the depression quotient as follows: the quotient of the maximum drop of the blood sugar (mg.%) (a) and the depression time (hours) (b).

At this juncture, the selection of the lowest blood sugar level caused by insulin must be taken into consideration.

If the minimum blood sugar level shown on the curve (c) is regarded as the lowest blood sugar level caused by insulin, then the depression time (b') may delay correspondingly, and, accordingly, the depression quotient may diminish unreasonably.

I regard the blood sugar level (B), which reached first within 10 mg.% above the minimum value shown on the curve, as the lowest level caused by the insulin injection.

Such variation of the blood sugar within 10 mg.% appears to be quite within the limits of physiological fluctuations or experimental errors at the time of examination.

Thus, the greater the depression quotient is, the higher the insulin sensitivity.

The depression quotient ranges from 11.2 to 119.3 in Type II, and from 6.7 to 64.7 in Type I. In 3 cases of renal glycosuria it ranges from 3 to 18. In 2 healthy persons it is found to be 34 and 40, respectively.

For convenience' sake, therefore, the whole series are classified by this depression quotient as follows: more than 61 as insulin sensitive, 40–31 as normal and less than 30 as insulin resistant.

According to this classification 49% of the total cases of diabetes mellitus were found to be insulin sensitive, 49% normal and 36% resistant. Furthermore, 56% of Type II were found to be insulin sensitive and 22% of this type insulin resistant; on the contrary, 30% of Type I were found to be insulin sensitive and 55% insulin resistant (Table III).

There is no close relation between the depression quotient and the age or the blood pressure, but a direct relation exists between the depression quotient and the quotient of weight and length of the body (Tab. IV). In general, the lean diabetic is more sensitive to insulin and the obese is more resistant.

Finally, as shown in Table V, the spontaneous blood sugar depression curve was followed as a control. In all but one, the blood sugar dropped spontaneously, whether large or small.

The corrected depression quotient, therefore, is measured as follows: instead of the maximum drop of the blood sugar a difference between

TABLE III

Depression Quotient and Type of Diabetes

| Type of diabetes | Case | Depression quotient | | | |
|---|---|---|---|---|---|
| | | Above 61 | 60—41 | 40—31 | Below 30 |
| Type II | Number | 7 | 8 | 6 | 6 |
| | % | 56 | | 22 | 22 |
| Type I | Number | 2 | 4 | 3 | 11 |
| | % | 30 | | 15 | 55 |
| Total | Number | 9 | 12 | 9 | 17 |
| | % | 45 | | 19 | 36 |
| Renal glycosuria | Number | | | 1 | 2 |
| Healthy persons | Number | | | 2 | |

TABLE IV

Depression Quotient and Quotient between Weight and Length

| Quotient between body weight and body length | Depression quotient | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Above 101 | 100—91 | 90—81 | 80—71 | 70—61 | 60—51 | 50—41 | 40—31 | 30—21 | 20—11 | 10—0 |
| 45—41 | | | | | | | | | 1 | 2 | 1 |
| 40—36 | | | | | | | 2 | 3 | 4 | 1 | |
| 35—31 | 1 | | | 1 | 2 | | 2 | 4 | 3 | 2 | |
| 30—26 | | | | | 1 | 3 | 3 | 3 | 2 | | |
| 25—21 | | | | | | 1 | 2 | 1 | 2 | 1 | |
| 20—16 | 1 | | | | 2 | 1 | | | | | |

it and the spontaneous drop in the same time is used in the calculation of the depression quotient.

Judging by the depression quotient and the corrected quotient no marked change was found in the insulin sensitivity, except 3 cases, which showed a change from normal sensitivity to resistance, in spite of the fact that the corrected quotient generally was reduced.

The spontaneous drop of the blood sugar as a control, therefore, may be dispensable, so long as the depression quotient is utilized.

TABLE V

Blood Sugar Depression After Insulin Injection and Spontaneous Depression

| Type | No. | Sex | Age | Interval of both tests (days) | Remarks | Initial Blood sugar value (mg.%) | Depression magnitude (mg.%) | Depression time (hours) | Depression quotient | Corrected depression quotient |
|---|---|---|---|---|---|---|---|---|---|---|
| Type II | 3 | ♀ | 17 | 6 | Insulin | 381 | 305 | 4½ | 67.8 | 48.2 |
| | | | | | Spontan. | 368 | 88 | — | — | — |
| | 9 | ♀ | 29 | 3 | Insulin | 271 | 146 | 2½ | 58.4 | 59.2 |
| | | | | | Spontan. | 214 | +2 | — | — | — |
| | 10 | ♂ | 43 | 4 | Insulin | 271 | 127 | 5 | 25.4 | 12.2 |
| | | | | | Spontan. | 267 | 66 | — | — | — |
| | 12 | ♀ | 21 | 4 | Insulin | 253 | 143 | 4 | 35.8 | 27.5 |
| | | | | | Spontan. | 254 | 33 | — | — | — |
| | 17 | ♂ | 65 | 7 | Insulin | 228 | 157 | 1½ | 104.7 | 102.7 |
| | | | | | Spontan. | 233 | 3 | — | — | — |
| | 18 | ♀ | 27 | 9 | Insulin | 225 | 129 | 3½ | 36.9 | 20.9 |
| | | | | | Spontan. | 234 | 56 | — | — | — |
| | 22 | ♀ | 49 | 4 | Insulin | 206 | 88 | 2½ | 35.2 | 27.2 |
| | | | | | Spontan. | 178 | 20 | — | — | — |
| | 24 | ♀ | 69 | 7 | Insulin | 184 | 85 | 1½ | 56.7 | 45.3 |
| | | | | | Spontan. | 171 | 17 | — | — | — |
| | 27 | ♂ | 67 | 5 | Insulin | 155 | 51 | 3 | 17.0 | 14.3 |
| | | | | | Spontan. | 184 | 8 | — | — | — |
| Type I | 33 | ♂ | 75 | 10 | Insulin | 164 | 97 | 1½ | 64.7 | 54.7 |
| | | | | | Spontan. | 171 | 15 | — | — | — |
| | 35 | ♀ | 50 | 3 | Insulin | 157 | 98 | 2 | 49.0 | 44.0 |
| | | | | | Spontan. | 160 | 10 | — | — | — |
| | 39 | ♀ | 16 | 3 | Insulin | 134 | 44 | 2 | 22.0 | 13.5 |
| | | | | | Spontan. | 104 | 17 | — | — | — |
| | 42 | ♂ | 55 | 3 | Insulin | 122 | 38 | 1½ | 25.3 | 24.7 |
| | | | | | Spontan. | 125 | 1 | — | — | — |
| | 43 | ♀ | 20 | 20 | Insulin | 115 | 30 | 1½ | 60.0 | 50.0 |
| | | | | | Spontan. | 121 | 5 | — | — | — |
| | 44 | ♂ | 54 | 2 | Insulin | 115 | 14 | 1 | 14.0 | 9.0 |
| | | | | | Spontan. | 106 | 5 | — | — | — |
| Healthy person | 51 | ♂ | 26 | 2 | Insulin | 102 | 17 | ½ | 34.0 | 32.0 |
| | | | | | Spontan. | 78 | 1 | — | — | — |

## DISCUSSION

As stated at the beginning, there are numerous investigations which have proposed various methods for the determination of the insulin sensitivity in diabetes mellitus.

Among them, the examination of the blood sugar curves after the insulin injection seems to be the simplest method for this purpose. However, so far as the magnitude of the blood sugar depression alone is aimed at, it can not be regarded as an appropriate method to estimate the insulin sensitivity.

I measured newly the depression quotient, produced from the depression magnitude and the depression time. According to this quotient 56% of Type II diabetics was found to be insulin sensitive and 22% of this type insulin resistant; on the contrary, 30% of Type I was found to be insulin sensitive and 55% insulin resistant. 45% of the total cases of diabetes mellitus was found to be insulin sensitive, 19% normal and 36% resistant.

In comparison with the statistics of Fenz[13] in Falta's Clinic, in my investigation the insulin sensitive diabetics are found to increase in number, on the contrary, the insulin resistant are found to decrease in number. These differences appear to be greater, when the Fenz's statistics are compared with my results found in Type II.

Though the methods of estimation are different, it seems that the distribution of insulin sensitivity in diabetic patients differs at home and abroad.

Besides, as shown in the results, a part of diabetics belonging to Type II, was actually treated with insulin application, in spite of the insulin resistance according to the depression quotient. On the contrary, a part of Type I was recovered with our diabetic diet therapy alone, in spite of the sensitivity according to the quotient.

For these reasons, I cannot agree with the conception that insulin therapy is unnecessary because of the insulin resistance measured by the examination in these cases. I would like to support Wislicki's opinion[14] that insulin therapy is as necessary as usual even in such cases, if they show high blood sugar and great urine sugar.

Bodansky and Simpson[15] found in healthy persons that the lean person was sensitive to insulin and the obese was resistant. The present work revealed similar results even in diabetic persons.

After Schmidt's example,[16] Leszler[17] classified diabetics from somatic type in 3 forms; lean (24%), intermediate (36%) and obese (40%). His classification appears to have some analogy with mine based on the depression quotient, though the incidence of the insulin sensitive diabetics in my classification occurs relatively greater than that of the lean diabetics in his classification.

## Conclusions

1. In order to observe the insulin sensitivity, the blood sugar curves after subcutaneous insulin injection were studied in diabetes mellitus,

containing 20 cases of Type I and 27 cases of Type II.

2. The higher the level of the initial blood sugar is, the greater the magnitude of the depression curve and the greater the depression time are.

3. As a criteria for the estimation of insulin sensitivity the depression quotient was measured anew.

4. According to this quotient 56% of Type II was found to be insulin sensitive and 22% of this type insulin resistant, on the contrary, 30% of Type I was found to be insulin sensitive and 55% insulin resistant.

5. There was a direct relation between the depression quotient and the quotient of weight and length of the body. The lean diabetic is sensitive to insulin and the obese is resistant.

6. As a control, the spontaneous blood sugar depression in starvation was discussed.

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# The Effect of Dietary Treatment on Insulin Sensitivity in Diabetes Mellitus

By

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There are many references which reveal that the increase in insulin sensitivity occurs in high carbohydrate and low fat diet; i.e. in rats by Abderhalden and Wertheimer,[1] Grevenstuk, Jongh and Laqueur,[2] Bainbridge,[3] in rabbits by Tiitso,[4] in hypophysectomized dogs by Heinbecker, Somogyi and Weichselbaum,[5] in healthy persons by Himsworth,[6][7] Wesselow and Griffiths,[8] in diabetic bodies by Mac Gillvray and Wagner,[9] Nadler,[10] etc.

However, in 1938, Card[11] concluded that the capacity to utilize increased carbohydrate shown by certain diabetics when transferred to a higher diet bears no constant relation to the phenomenon studied in the insulin depression curve.

In the previous paper,[12] I studied the blood sugar curves after insulin injection in diabetes mellitus and measured the depression quotient as criteria for the estimation of insulin sensitivity, a new index contrived by me.

In this paper I intend to report further the effect of dietary treatment on insulin sensitivity, measured repeatedly before and after the treatment by this quotient.

## Experimental

16 cases of diabetes mellitus were examined, 5 of which belonged to Type I, remaining 11 to Type II.

Methods and others are the same as in the previous paper.[12]

## Results

For each patient the first examination was performed at his admission to our Clinic; i.e., in the preliminary free diet period or at the beginning of dietary treatment.

The second examination was performed in convalescence, after dietary treatment with or without insulin application.

The interval between both examinations ranges from 27 to 67 days in Type I and from 38 to 95 days in Type II.

Results are given in Table I.

TABLE I

Change of Insulin Sensitivity After Treatment

| Type | No. | Sex | Age | Interval of both tests (days) | Remarks (Before or after treatment) | Initial blood sugar value (mg.%) | Depression magnitude (mg. %) | Depression time (hours) | Depression quotient | Group divided by the results |
|---|---|---|---|---|---|---|---|---|---|---|
| Type II | 1 | ♂ | 42 | 72 | Before | 407 | 214 | 5 | 42.8 | Group A |
| | | | | | After | 245 | 109 | 3½ | 31.1 | |
| | 2 | ♂ | 14 | 71 | Before | 322 | 225 | 3 | 75.0 | |
| | | | | | After | 125 | 71 | 1 | 71.0 | |
| | 3 | ♂ | 46 | 48 | Before | 312 | 126 | 4 | 31.5 | |
| | | | | | After | 217 | 62 | 2 | 31.0 | |
| | 4 | ♂ | 44 | 39 | Before | 291 | 183 | 4 | 45.8 | |
| | | | | | After | 193 | 80 | 2½ | 32.0 | |
| | 5 | ♀ | 25 | 62 | Before | 273 | 95 | 4½ | 21.1 | |
| | | | | | After | 169 | 35 | 1½ | 23.3 | |
| | 6 | ♂ | 43 | 55 | Before | 271 | 127 | 5 | 25.4 | |
| | | | | | After | 215 | 77 | 3 | 25.7 | |
| | 7 | ♀ | 62 | 49 | Before | 256 | 157 | 3½ | 44.9 | Group B |
| | | | | | After | 183 | 132 | 1½ | 88.0 | |
| | 8 | ♀ | 21 | 95 | Before | 253 | 143 | 4 | 35.8 | |
| | | | | | After | 158 | 106 | 2 | 53.0 | |
| | 9 | ♂ | 57 | 71 | Before | 219 | 118 | 4½ | 24.0 | |
| | | | | | After | 139 | 69 | 1½ | 46.0 | |
| | 10 | ♂ | 37 | 86 | Before | 219 | 51 | 4½ | 11.2 | |
| | | | | | After | 131 | 77 | 1 | 77.0 | |
| | 11 | ♀ | 49 | 74 | Before | 206 | 88 | 2½ | 35.2 | |
| | | | | | After | 138 | 74 | 1 | 74.0 | |
| Type I | 12 | ♂ | 57 | 27 | Before | 205 | 144 | 3 | 48.0 | Group C |
| | | | | | After | 104 | 33 | 2 | 16.5 | |
| | 13 | ♂ | 51 | 29 | Before | 197 | 94 | 3½ | 26.9 | |
| | | | | | After | 118 | 36 | 1 | 36.0 | |
| | 14 | ♂ | 58 | 68 | Before | 181 | 82 | 3 | 27.3 | |
| | | | | | After | 191 | 60 | 2½ | 24.0 | |
| | 15 | ♀ | 64 | 67 | Before | 164 | 89 | 3 | 29.7 | |
| | | | | | After | 176 | 67 | 2 | 33.5 | |
| | 16 | ♂ | 56 | 56 | Before | 102 | 10 | 1½ | 6.7 | |
| | | | | | After | 89 | 9 | 2 | 4.5 | |

Both the magnitude of the blood sugar depression and the depression time diminish markedly in convalescence in all the cases except one. It is easily conceivable that they decrease in proportion to the fall in the initial blood sugar in convalescence.

On the contrary, changes in depression quotients show interesting results. The cases are divided into 3 groups as follows:—group A contains Cases No. 1–6, corresponding to severe cases of diabetes mellitus of Type II with initial blood sugar above 250 mg.%; group B contains Cases No. 7–11, corresponding to mild cases of Type II with initial blood sugar below 250 mg.% and group C contains Cases No. 12–16, corresponding to Type I.

After treatment the depression quotients showed no change in the group A and C, although they increased markedly in the group B.

Though the insulin sensitivity remained unchanged in the severe cases of Type II and in the majority of Type I notwithstanding the recovery from diabetic symptoms, the majority of mild cases of Type II showed an increase in it.

Furthermore, the change in the corrected depression quotient, in which the spontaneous blood sugar depression was taken into consideration, as stated before, was examined in 3 cases of Type II (Table II).

TABLE II

Change of Insulin Sensitivity in Consideration with Corrected Depression Quotient

| No. of case | Sex | Age | Before or after treatment | After insulin or spontan. | Interval of both tests (days) | Initial blood sugar value (mg. %) | Depression magnitude (mg. %) | Depression time (hours) | Depression quotient | Corrected depression quotient |
|---|---|---|---|---|---|---|---|---|---|---|
| 6 | ♂ | 43 | Before | Insulin | 1 | 271 | 127 | 5 | 25.4 | 12.2 |
| | | | | Spontan. | 4 | 267 | 66 | — | — | — |
| | | | After | Insulin | 99 | 215 | 77 | 3 | 25.7 | 13.3 |
| | | | | Spontan. | 100 | 188 | 37 | — | — | — |
| 8 | ♀ | 21 | Before | Insulin | 1 | 253 | 143 | 4 | 35.8 | 27.5 |
| | | | | Spontan. | 4 | 254 | 33 | — | — | — |
| | | | After | Insulin | 91 | 158 | 106 | 2 | 53.0 | 37.5 |
| | | | | Spontan. | 92 | 188 | 31 | — | — | — |
| 11 | ♀ | 49 | Before | Insulin | 1 | 206 | 88 | 2½ | 35.2 | 27.2 |
| | | | | Spontan. | 4 | 178 | 20 | — | — | — |
| | | | After | Insulin | 73 | 138 | 74 | 1 | 74.0 | 65.0 |
| | | | | Spontan. | 75 | 136 | 9 | — | — | — |

Changes in quotients before and after treatment occurred correspondingly with each other. Judging either by the depression quotient

or by the corrected quotient, the changes in insulin sensitivity, therefore, would be about the same.

From this point of view, also, the depression quotient seems to be adequate in discussing the blood sugar depression following insulin injection.

## Discussion

As stated at the beginning of this paper, there are many authors who stated that the insulin sensitivity was influenced by diet, especially by high carbohydrate and low fat diet.

When the insulin sensitivity is measured by the magnitude of the blood sugar depression after insulin injection, it appears to be the natural result that they diminish in proportion to decrease by the treatment.

As stated in the previous paper, I have used the depression quotient for measuring of the insulin sensitivity. By means of this quotient the insulin sensitivity appears to remain stable in the severe cases of diabetes mellitus of Type II (group A) and in about total cases of Type I (group C) after treatment, notwithstanding the recovery from diabetic symptoms. On the contrary, in the mild cases of Type II (group B) it increases markedly.

It is conceivable that in such severe cases as group A the insulin sensitivity, whether sensitive or resistant, is not affected by treatment, even though accompanied with insulin.

Differently, the constancy in Type I is due to small insulin sensitivity, and accordingly to its small extent of the movability.

On the contrary, in mild cases of Type II, the insulin sensitivity appears to increase in convalescence, presumably as an effect of treatment with high carbohydrate and low fat diet.

## Conclusions

1. In order to observe the effect of dietary treatment on insulin sensitivity the blood sugar curves after insulin injection were estimated repeatedly in 16 cases of diabetes mellitus, for which the depression quotient was measured as criteria.

2. Although the insulin sensitivity remained unchanged in the severe cases of Type II notwithstanding the recovery from diabetic symptoms, the majority of mild cases of this type showed an increase in it, presumably as an effect of treatment with carbohydrate rich diet.

3. Many cases of Type I revealed no change.

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